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Interleukin 6 trans-signalling

Cardiovascular risk marker or therapeutical target?

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Til min mor, Jonna. Du er altid hos mig.

”Livet forstås baglæns, men må leves forlæns”

Søren Kierkegaard 1843

ABSTRACT

Background: Interleukin (IL) 6 signals in two pathways. In classical signalling, essential in antimicrobial defence and tissue regeneration, IL6 binds the membrane-bound IL6 receptor (IL6R) which associates with the signal transducing receptor gp130. The pro-inflammatory effects of IL6 on the other hand, are governed by IL6 trans-signalling via the binary complex of IL6 and the soluble IL6R (sIL6R) through gp130 binding. The binary IL6:sIL6R complex is neutralised by the soluble gp130 (sgp130) thereby preventing IL6 trans-signalling. IL6 trans-signalling is associated with chronic inflammatory conditions.

Overarching aim: To analyse the association of IL6 trans-signalling, estimated by a ratio between the active binary IL6:sIL6R complex and the inactive ternary IL6:sIL6R:sgp130 complex (the binary/ternary complex ratio [B/T ratio]), with the risk of cardiovascular event (CVE) and investigate the presence of IL6 signalling in manifest atherosclerosis.

Methods and results

Study I: In a prospective cohort of 60-year-olds (60YO) without prevalent cardiovascular disease (CVD) (n=3645), the binary IL6:sIL6R and ternary IL6:sIL6R:sgp130 complex levels, expressed in nanomole/litre, were derived from baseline serum concentrations of IL6, sIL6R, and sgp130. IL6 trans-signalling was estimated by a ratio between the pro-inflammatory binary complex and the inactivated ternary complex, the B/T ratio. Cox regression was used to assess the risk of CVE (myocardial infarction, angina pectoris and ischaemic stroke), expressed as hazard ratio (HR) with 95% confidence interval (CI), associated with increasing circulating levels of IL6, sIL6R, sgp130 and with the B/T ratio, the latter analysed both as a continuous variable and dichotomised at the median. Estimates were adjusted for the common cardiovascular risk factors. The discriminatory ability of the B/T ratio as predictor was assessed by the area under the curve (AUC) and net reclassification index (NRI) in relation to the Framingham risk score (FRS) and IL6.

The B/T ratio >median associates with increased CVE risk (adjusted HR 1.44, 95% CI 1.21–1.72). The prediction of CVE improved by adding the B/T ratio to the FRS and IL6 and 10% of subjects were re-classified.

Study II: In the 60YO cohort, CVE risk associated with B/T ratio >median was investigated in subjects with low-intermediate cardiovascular risk defined by LDL ≤ / >4.0 mmol/L or according to the FRS. The difference in time to event (years; 95% CI) was analysed with quantile regression. In secondary analyses, risk of coronary and cerebrovascular events and time to event was analysed. Biological interaction between LDL and B/T ratio was estimated on the additive scale and the incremental discriminatory value of the B/T ratio with FRS and IL6 was compared in subjects with LDL≤ and >4.0 mmol/L.

B/T ratio was associated with an increased risk of CVE primarily the LDL≤4.0 group (adjusted HR 1.59; 95% CI 1.24-2.05) but also in FRS <20% 10-year risk. The highest risk and earliest events were seen for ischemic stroke. No interaction between LDL and the B/T ratio was seen and the B/T ratio improved prediction in the LDL ≤4.0 group.

Study III: Carotid artery plaques were obtained during endarterectomy in patients with high-grade carotid artery stenosis in the Biobank of Karolinska Endarterectomies (BiKE) study. Oligoprimers were designed to selectively amplify *IL6R*, *sIL6R*, *GP130* and s*GP130* genes. Using cDNA reverse transcribed from RNA extracted from plaques quantitative real time-PCR was performed and the relative difference in expression between groups was estimated using the ΔCT method (n=78). Correlations between plaque gene expression and plasma levels were tested using Spearman's correlation coefficient.

Gene expression of *IL6*, *IL6R*, *sIL6R*, *GP130* and *sGP130* were detected in all plaques. A pattern of differential plaque expression depending on disease severity was seen as well as a trend towards positive correlations between IL6 and sIL6R plaque expression and corresponding protein levels in the circulation.

Study IV: In the 60YO cohort, risk of incident ischemic stroke associated with the B/T ratio >median was analysed using Cox regression and stratified by prevalent or incident atrial fibrillation (AF). In secondary analyses, the risk of first-time diagnosis of AF associated with the B/T ratio >median was analysed.

The B/T ratio was associated with ischemic stroke risk only in subjects without AF (adjusted HR 1.49; 95% CI 1.08-2.06). In addition, no association between the B/T ratio and risk of first-ever AF (HR 0.96; 95% CI 0.75-1.24) was seen albeit an indication of an association with IL6.

Conclusions: Pro-inflammatory IL6 trans-signalling, estimated by B/T ratio>median mirroring a relative excess of the binary complex (IL6:sIL6R) in relation to the inactive ternary complex (IL6:sIL6R:sgp130), is associated with an increased risk of future CVE in subjects without prevalent CVD, primarily in individuals at low-intermediate risk of CVE. IL6 signalling is present in carotid artery plaques and the B/T ratio is associated with an increased risk of atherothrombotic ischemic stroke and early stroke events albeit no association was established for ischemic stroke in relation to AF or for AF per se.

LIST OF SCIENTIFIC PAPERS

I. **Ziegler L**, Gajulapuri A, Frumento P, Bonomi A, Wallén H, de Faire U, Rose-John S, Gigante B. Interleukin 6 trans-signalling and risk of future cardiovascular events. *Cardiovascular Research*. 2019;115:213-221

II. **Ziegler L**, Frumento P, Wallén H, de Faire U, Gigante B. The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events. *European Journal of Preventive Cardiology*. 2020;27:122-129

III. **Ziegler L**, Lundqvist J, Dreij K, Wallén H, de Faire U, Paulsson-Berne G, Hedin U, Matic L, Gigante B. Expression of IL6 signaling receptors in carotid atherosclerosis. *Manuscript*.

IV. **Ziegler L**, Wallén H, Aspberg S, de Faire U, Gigante B. IL6 trans-signalling associates with atherothrombotic but not with cardioembolic stroke. *Manuscript.*

LIST OF ABBREVIATIONS

60YO	The cohort of 60-year-olds
AF	Atrial fibrillation
ATC	Anatomic Therapeutic Chemical classification system
AUC	Area under the receiver-operating characteristics curve
BiKE	Biobank of Karolinska Endarterectomies
BMI	Body mass index
CAD	Coronary artery disease
CEA	Carotid endarterectomy
CHIP	Clonal hematopoiesis of indeterminate potential
CI	Confidence interval
CNS	Central nervous system
CRP	C-reactive protein
CT	Computed tomography
CVD	Cardiovascular disease
CVE	Cardiovascular events
ECG	Electrocardiogram
FDA	Federal Drug Administration
FRS	Framingham risk score
gp130	Glycoprotein 130
GWAS	Genome-wide association study
HR	Hazard ratio
hsCRP	High-sensitivity C-reactive protein
IBD	Inflammatory bowel disease
ICD-10	International Classification of Diseases 10th revision
IL	Interleukin
IL6R	Interleukin 6 receptor
IQR	Interquartile range
JAK	Janus kinases
kDa	Kilo Dalton

LDL	Low density lipoprotein
M	Molar
MAPK	Mitogen–activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
mCRP	Monomeric CRP
MI	Myocardial infarction
NLRP3	Nucleotide-binding domain and leucine-rich repeat containing protein 3
nM	Nano molar
NRI	Net reclassification index
oxLDL	Oxidised low density lipoprotein
PCR	Polymerase chain reaction
pg	Pico gram
pM	Pico molar
RA	Rheumatoid arthritis
ROC	Receiver-operating characteristics
semi-qRT-PCR	Semi-quantitative real time PCR
sgp130	Soluble glycoprotein 130
sIL6R	Soluble interleukin 6 receptor
SMC	Smooth muscle cells
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
STAT	Signal transducer and activator of transcription
Th cell	T helper cell
TIA	Transitory ischemic attack
Treg	T regulatory cell

CONTENTS

1 BACKGROUND

1.1 Cardiovascular diseases: epidemiological notes

Improved public health and advances in medical care have succeeded in reducing cardiovascular disease (CVD) mortality and the age-standardised death rate has fallen during the past 30 years in high-income countries and to some extent in middle-income countries (1). Nevertheless, CVD is the leading cause of death in the world accounting for 15 million deaths in 2016 according to the World Health Organisation (2).

Myocardial infarction (MI) is the first and stroke the second most common cause of death worldwide (1, 2). In Sweden, cardiovascular mortality constitutes 33% of all deaths (3). In 2017, there were 26 000 cases of MI and nearly 21 000 cases of stroke in 2018 (4, 5). The vast majority of strokes (87%) are ischemic and 21% have atrial fibrillation (AF) as a potential cause of cerebral emboli (4). In line with the trend in high-income countries, numbers of acute MI and stroke have decreased in Sweden (5, 6).

Increased awareness of cardiovascular health in the population has resulted in a decline in the number of smokers, attention is paid to avoid a sedentary lifestyle, to promote physical activity and to eat a balanced diet. Together with effective secondary preventive treatments, these factors have contributed to improve CVD statistics albeit challenges such as increasing incidence of obesity and diabetes mellitus type 2, known cardiovascular risk factors, remain to be conquered (7).

We stand at the breaking point of the next paradigm in CVD prevention. In the last decade, the understanding of CVD risk has begun to shift from a primary-secondary preventive approach to more individualised prediction with the aim to assess individual cardiovascular risk on a continuous risk spectrum (8). To increase the ability to predict and cure CVD and reduce the burden of CVD, we need novel biomarkers able to identify individuals at risk, above and beyond the known cardiovascular risk factors and risk scores.

1.2 Inflammation in atherosclerosis

Atherosclerosis is the hallmark of coronary and cerebrovascular disease and inflammation the soil in which atherosclerotic lesions arise. In the coronary and carotid vascular beds, atherosclerotic plaques are the cause underlying coronary artery disease (CAD) and ischemic stroke, albeit the mechanisms might be somewhat different (9, 10). A large bulk of evidence supports the notion that a low-grade chronic inflammation in atherosclerosis contributes to and interacts with the traditional cardiovascular risk factors; hypertension, diabetes mellitus, hyperlipidaemia,

obesity, sedentary lifestyle and smoking, in determining the cardiovascular risk in men and women (11). Recently, a large clinical trial demonstrated that in patients with stable CAD, treating inflammation reduces the risk of future cardiovascular events (CVE) (12). This strongly suggests that inflammation partly explains the cardiovascular risk remaining when treating or adjusting for the traditional cardiovascular risk factors, the so called residual inflammatory risk (13).

Much effort has been put into the quest to find both novel inflammatory targets to improve treatment of atherosclerosis related CVD and inflammatory biomarkers able to ameliorate our ability to identify individuals at risk of future CVE.

In this thesis, we analyse the predictive capacity of a novel inflammatory biomarker, assessing interleukin 6 (IL6) trans-signalling activity, and its role in predicting the risk of CAD and ischemic stroke above and beyond the known cardiovascular risk factors. We also discuss if targeting IL6 trans-signalling might represent a novel treatment moiety for CVD.

1.2.1 The central axis of inflammation in atherosclerosis

The inflammatory response in atherosclerosis is mainly driven by the central axis of inflammation: the interleukin (IL)1β-IL6-C-reactive protein (CRP) pathway (**Figure 1**). IL6 signals through two signalling systems: the so called IL6 classical signalling with CRP as one of the end-products (**Figure 1**, left hand side) and the so called IL6 trans-signalling (**Figure 1**, right hand side).

Figure 1. Schematic representation of the IL1β-IL6-CRP pathway

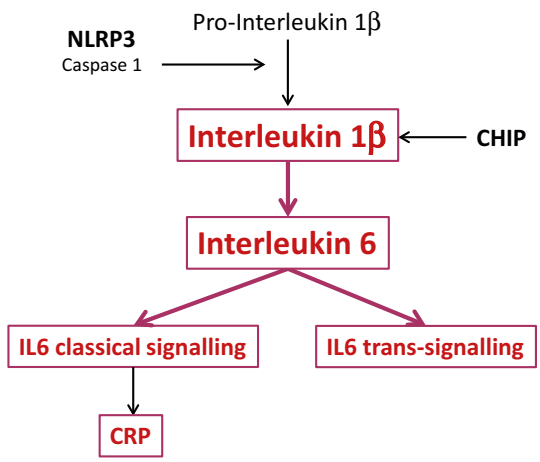

Figure 1. *NLRP3=(NOD [nucleotide oligomerisation domain]-LRR [leucine rich repeat]- and PYD [pyrin domain]-containing protein 3) inflammasome, CHIP=clonal hematopoiesis of Indeterminate Potential, CRP=C-reactive protein. Ziegler 2020 (unpublished).*

1.2.1.1 Interleukin 1β

Interleukin 1β (IL1β) is the upstream regulator of the central axis of inflammation.

In atherosclerotic plaques, IL1β synthesis is tightly regulated: pro-IL1β, the inactive IL1β precursor, is produced intra-cellularly and activated via proteolytic cleavage by the protease Caspase-1 which in turn needs to be activated by the NLRP3 (NOD [nucleotide oligomerisation domain]-LRR [leucine rich repeat]-and PYD [pyrin domain]-containing protein 3) inflammasome (14). Inflammasomes are intracellular multimolecular signalling complexes possessing the ability to activate innate immune system responses (15). The NLRP3 inflammasome requires a two step activation in which the first step induces the transcription of the NLRP3 constituents as well as the pro-IL1β and the second step activates the assembly of the inflammasome and subsequently the cleavage of pro-IL1β into the active IL1β (14). Cholesterol crystals leaking out of dying foam cells in the necrotic core of atherosclerotic plaques, through inflammasome activation, elicit IL1β release which is associated with plaque progression in *in vitro* studies (16-18). The rigorously regulated activation of the NLRP3 can however be impaired due to mutations and give rise to auto-inflammatory disorders, rare diseases characterised by recurrent fever and local inflammation in organs such as the skin and joints (19).

Another mechanism known to regulate IL1β production is the clonal haematopoiesis of indeterminate potential (CHIP), a precancerous condition well known from studies of haematological malignancies, in which somatic mutations allow competitive growth and clonal expansion of stem cells (20). This phenomenon, associated with aging and present in 10% of individuals >70 years, has recently been demonstrated to increase the risk of cardiovascular mortality (21, 22). The CHIP mutations are associated with a pro-inflammatory phenotype and *TET2*, a transcriptional regulator affecting the NLRP3 inflammasome mediated IL1β secretion and enhancing atherosclerotic lesions progression, is among the most frequent CHIP mutations (23, 24).

Hence, biological data indicate that IL1β may represent a potential target for treatment. This hypothesis was tested and confirmed by the results of the CANTOS study, a double blind randomised clinical trial demonstrating that treatment with canakinumab, a human monoclonal antibody against IL1β , was associated with a 15% relative CVE risk reduction in patients with chronic CAD (12). IL1β is difficult to measure and clinical studies analysing IL1β inhibition such as the CANTOS study, have measured IL6 and/or CRP to assess activity in the pathway instead.

1.2.1.2 C- reactive protein

CRP, a nonglycosylated pentraxin circulating in the blood arranged in a pentamer, was first described in the 1930ies and later found to be an acute phase reactant. In

the late nineties, it was demonstrated that levels of high sensitivity CRP (hsCRP) were elevated decades before the first ever CVE (25, 26). Based on these results P.M. Ridker and collegues demonstrated that adding CRP and parental history of CVD to the Framingham risk score (FRS), improved risk classification measures and individuals at low cardiovascular risk were correctly re-classified as at intermediate risk (27, 28). A large metanalysis led by the Emerging Risk Factor Consortium, supported this notion showing a robust association between increased hsCRP and risk of future CVE (29). By then, the JUPITER trial had demonstrated that reducing cholesterol levels with a cholesterol lowering drug (rosuvastatin) also had beneficial anti-inflammatory effects and reduced the rate of first ever MI and stroke by half (30). The study supported the concept of "dual targets" i.e. achieving the greatest effect on CVD when both low-density lipoprotein (LDL) cholesterol and CRP are significantly lowered, a finding supported also by the PROVE IT and the A to Z clinical trials (31, 32). On the other hand, two population-based Mendelian randomisation studies challenged the notion of a causal association of CRP with CVD and found that genetic variants associated with CRP levels were not associated with the risk of CAD (33, 34).

The discovery of a monomeric CRP (mCRP) molecule is relatively new (35). The mCRP stems from the pentameric CRP when it, under certain conditions such as in contact with activated platelets, dissociates into a singular pentraxin monomers (36). The mCRP has been demonstrated in atherosclerotic plaques and recently mCRP was found to circulate in the blood stream after conversion from pentameric to monomeric form on microparticles in patients with MI (36, 37). The discovery of mCRP and its association with atherosclerosis can potentially shed a light on the complex association between CRP and CVD. At present, the existing data support the use of hsCRP as a biomarker for cardiovascular risk but, given the fact that causality of the association between CRP and CVD risk is not proven, CRP is unfit as an interventional target for treament and prevention (33, 34).

1.3 Interleukin 6

In 1968, the discovery that T cells were interacting with B cell antibody synthesis led to the hypothesis that signalling molecules derived from T cells were able to stimulate B cells (38). One of these molecules was interleukin 6 (IL6), a 184 amino acids long protein possessing pleiotropic properties which initially confused researchers into believing they were carried out by different signalling molecules. IL6, in fact, has anti-inflammatory as well as pro-inflammatory properties depending on which IL6 signalling pathway is active as will be descibed in detail below (39).

IL6 belongs to the IL6 family of cytokines (including e.g. IL11, ciliary neurotrophic factor, leukaemia inhibitory factor, oncostatin M, cardiotrophin 1, cardiotrophin-like cytokine, and IL27) that share similar biological properties (40). In particular, they all signal via receptor complexes consisting of an individual ligand binding α-receptor and a shared signal transducing β-receptor, glycoprotein 130 (gp130) (41). IL6 is synthesised by a large spectrum of cells: hepatocytes, cardiomyocytes, lipocytes, immune cells including monocytes and macrophages and vascular wall cells such as endothelial cells, smooth muscle cells (SMC) and fibroblasts (42). Once released in the circulation, IL6 circulates in extremely low concentrations, as reported in **Table 1**. The half-life of IL6 depends on the physiological and pathophysiological condition in the human body and ranges from <10 minutes to 2-15 hours (43-45).

Table 1. Circulating levels of IL6, sIL6R and sgp130 and dissociation constants for complex binding in IL6 classical and trans-signalling

	sIL6R (30–70 ng/mL)	**IL6R**
IL6 (1–5 pg/mL)	1 nM	1 nM
	sgp130 (200–400 ng/mL)	**gp130**
IL6:(s)IL6R	10 pM	10 pM

The circulating serum concentrations of IL6, sIL6R and sgp130 are reported within parenthesis in g/mL while dissociation constants for the binding of IL6 to the (s)IL6R and of the binary IL6:sIL6R complex to (s)gp130 are reported in molar=M.

1.3.1 Interleukin 6 signalling

IL6 signalling occurs through the binding of IL6 to the ligand binding IL6 receptor (IL6R) also known as IL6Rα or glycoprotein (gp) 80. The IL6R exists in two isoforms: a membrane-bound form and a soluble one (sIL6R). IL6 binds to IL6R with the same affinity regardless of the receptor isoform (**Table 1**) (46, 47).

To elicit the intracellular signalling cascade, the IL6:(s)IL6R complex associates with the signal transducing co-receptor gp130 (also known as IL6Rβ or CD130). Neither IL6 nor IL6R can bind gp130 on its own, thus the binary complex of IL6 and (s)IL6R is needed for the binding to gp130 (48). The IL6:(s)IL6R binds the signal transducing β-receptor gp130 with high affinity as seen in **Table 1** (49). This affinity gradient, with lower affinity between the ligand and the ligand binding receptor (IL6:(s)IL6R) and higher affinity in the binding of the binary IL6:(s)IL6R complex to the signal transducing receptor gp130**,** is shared with the other cytokines in the IL6 cytokine family (46, 50).

The expression of the membrane-bound IL6R is limited to hepatocytes, hematopoietic cells, immune cells and endothelial cells whereas gp130 is expressed on all human cells (51, 52).

The signalling moiety mediated by the binding of IL6:IL6R to gp130 is known as IL6 classical signalling and exists only on cells expressing the membrane-bound IL6R (53). The IL6 signal mediated by the IL6:sIL6R binding to the gp130 is known as IL6 trans-signalling. The circulating IL6 in complex with sIL6R enables IL6 trans-signalling to impact all cells of the body given the ubiquitous expression of gp130 (53).

1.3.2 Interleukin 6 signalling: intracellular mediators

Upon IL6 binding the (s)IL6R, the subsequent association with gp130 induces a homodimerisation of gp130 and the intracellular recruitment of the signal transducing tyrosine kinases, Janus kinases (JAK) which phosphorylates tyrosine residues on the gp130 receptor. The IL6 triggered JAK activation can follow either the JAK-SHP-2 (Src homology region 2 domain-containing phosphatase-2)-mitogen–activated protein kinase (MAPK) pathway or the JAK-STAT3 (signal transducer and activator of transcription 3) pathway (54) (**Figure 2**). In the latter, the most common IL6 induced pathway, JAK transduces the cytokine-mediated signal by docking STAT3 which after translocation into the nucleus binds the promoter region of target genes (41, 55). Upon binding to IL6R and gp30, the complex bound IL6 is rapidly internalised and eventually degraded while remaining IL6R on the cell membrane are down-regulated thus desensitising the cell to IL6 (49, 55). A special case of IL6 signalling is the endosomal intracellular autocrine signalling seen in immune system dendritic cells and hepatocytes (56, 57).

The suppressor of cytokine signalling (SOCS) family members 1 and 3 regulate the intracellular IL6 signalling via feedback regulation (58). The primary regulator, SOCS3 inhibits JAK activity when binding JAK and gp130 simultaneously (59). The importance of SOCS3 regulation is demonstrated by the observation that mice genetically engineered to have a defective binding of SOCS3 to gp130/JAK develop severe inflammatory conditions and cancer (59). In addition, IL6 gene expression is regulated by RNA binding proteins which either prolong or terminate expression. Imbalance in this regulation may result in overexpression of IL6 as seen in cancer and autoimmune diseases (60).

Figure 2. Schematic illustration of the intracellular IL6 signalling pathways

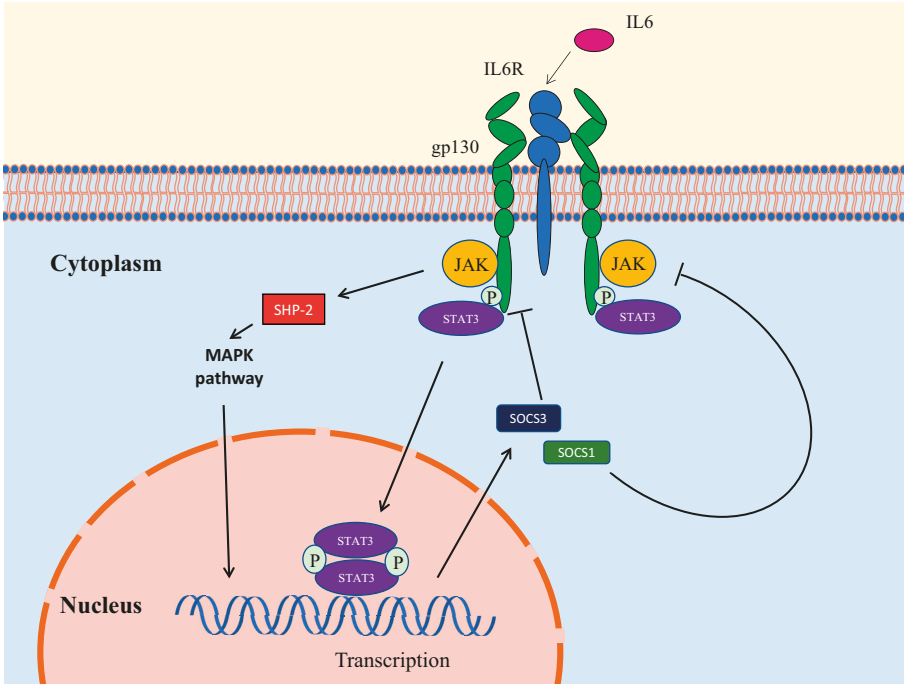

Figure 2. *MAPK=mitogen–activated protein kinase, SHP-2= Src homology region 2 domain-containing phosphatase-2, STAT3= signal transducer and activator of transcription 3, JAK=janus kinase, P=phosphate, SOCS1 and SOCS3=suppressor of cytokine signalling 1 and 3.*
Ziegler 2020 (unpublished)

1.3.3 Interleukin 6 classical signalling

The receptors of the IL6 classical pathway are depicted in **Figure 3**.

Overall the IL6 classical signalling possesses important physiological properties in the immune system and in cell regeneration. In the immune system, IL6 classical signalling is pivotal in the innate immune reactions i.e. the direct nonspecific cell mediated part of the immune response e.g. attracting monocytes and stimulating their differentiation into macrophages (61). In addition, IL6 classical signalling is active in the acquired antibody mediated adaptive immune system being a regulator of B cell transition to antibody-producing plasma cells and T cell differentiation (62, 63). IL6 stimulates the development of the pro-inflammatory T helper cell (Th) 17 while inhibiting the differentiation of anti-inflammatory regulatory T cells (Treg), an effect also mediated by IL6 trans-signalling (64, 65).

IL6 classical signalling is the main mediator of the acute phase reaction in the liver. The main effects are increasing levels of CRP, fibrinogen, haptoglobin, and serum amyloid A and simultaneously decreasing albumin and transferrin production (66).

As a consequence of the central role for IL6 in immunity, IL6 deficiency undermines the antibacterial defence in the immune system thus increasing susceptibility to infectious diseases. Mutations in genes encoding JAK and STAT3, such as in autosomal dominant hyper-IgE syndrome, result in Th17 deficiency with recurrent infections, primarily pneumonia (67, 68). In addition, when inhibiting IL1β with canakinumab the incidence of severe infections augmented (12).

When IL6, on the other hand, is produced in excess, such as in the inherited Castleman's syndrome, an imbalance between pro-inflammatory Th17 cells and anti-inflammatory Treg cells are inhibited with accompanying inflammation, autoimmunity, lymphadenopathy, and hyperglobulinemia as a result (60, 69). Moreover, experimental and clinical data point to a crucial role for IL6 as a mediator of antibody mediated tissue and organ transplant rejections (70).

IL6 classical signalling also exerts anti-inflammatory effects by stimulating the secretion of the IL1 receptor antagonist thus dampening signalling through the IL1β-IL6-CRP pathway (66). Moreover, IL6 classical signalling possesses regenerative, cytoprotective and wound healing properties in different organs and tissues such as the nervous and musculoskeletal system (40, 71-74).

Figure 3. Schematic presentation of IL6 classical and trans-signalling

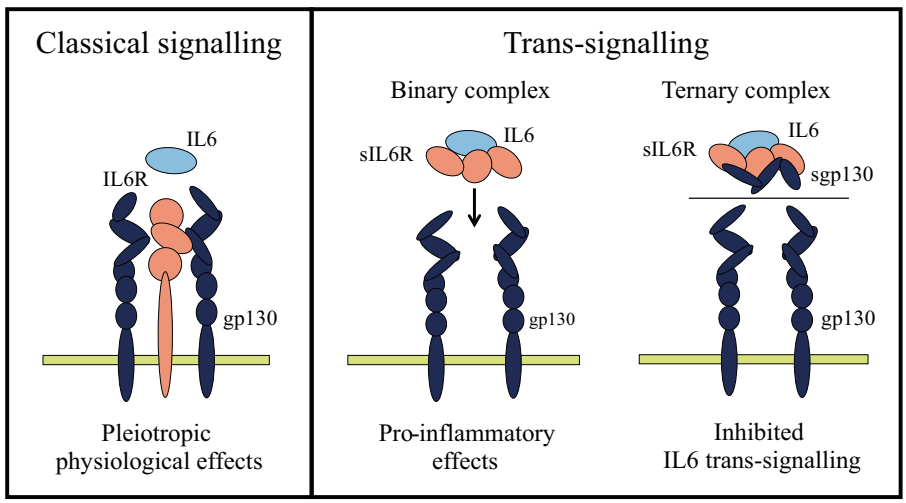

Figure 3. *Ziegler 2020 (unpublished)*

1.3.4 Interleukin 6 trans-signalling

IL6 trans-signalling is the IL6 signalling pathway responsible for the pro-inflammatory effects of IL6. In IL6 trans-signalling, IL6 binds the soluble isoform of the IL6R, the sIL6R (39). Subsequently the binary IL6:sIL6R complex binds the membrane-bound gp130 to elicit the intracellular signal cascade (**Figure 2**). This signalling moiety is tightly regulated to avoid uncontrolled systemic inflammation: a soluble isoform of gp130 (sgp130) functions as a natural inhibitor of IL6 trans-signalling by neutralising the excess of the binary IL6:sIL6R complex (75) (**Figure 3**).

1.3.4.1 The soluble interleukin 6 receptor

The sIL6R is produced mainly through shedding, i.e. limited proteolytic cleavage of the membrane-bound IL6R by the metalloproteinases ADAM17 and ADAM10 (76, 77). The receptor is cleaved at the juxtamembrane region (78). In acute inflammation, shedding of IL6R on neutrophils by ADAM 17 is elicited by inflammation associated factors such as apoptosis, bacterial toxins, CRP and IL8 (76, 79, 80). Levels of sIL6R are halved in IL6 knockout mice indicating the presence of an autocrine feedback loop of IL6 on IL6R expression (81).

Circulating levels of sIL6R are genetically regulated. A single nucleotide polymorphism (SNP) within the extracellular domain of the *IL6R* gene (rs2228145-AC) causes an amino-acid change (Asp358Ala) that favours shedding and is associated with a two-fold increase in sIL6R levels (82). The presence of the C-allele is not associated with the traditional cardiovascular risk factors but it has been consistently shown that for every copy of the C-allele inherited, the sIL6R concentration increased on average by 34% (83). Homozygotes for the rare allele at rs4537545-CT, a SNP in full linkage disequilibrium with rs2228145, exhibited doubled sIL6R concentrations as compared to homozygotes for the common allele (84). Moreover, the same study demonstrated that the *IL6R* SNP analysed accounted for 20% of the sIL6R variance in the circulation. A small proportion of the circulating sIL6R is produced by alternative splicing, a transcriptional mechanism that results in the trans-membrane part of the receptor not being translated (85-89).

1.3.4.2 The soluble glycoprotein 130

The sgp130 is the natural circulating antagonist of IL6 trans-signalling. The inhibitory properties of sgp130 consist in the ability to competitively sequester and neutralise the binary IL6:sIL6R complex (**Figure 3**). The binary IL6:sIL6R complex binds sgp130 with the same affinity as gp130 (90) (**Table 1**). The inactivated ternary IL6:sIL6R:sgp130 complex is a hexameric consisting of two molecules each of IL6, sIL6R and sgp130 (91). The addition of sgp130 to the binary complex prevents docking to gp130 hence inhibiting IL6 trans-signalling (90) (**Figure 3**). Little is known about the regulation and the physiological role of circulating levels of sgp130. The majority of the circulating sgp130 is produced by alternative splicing of the gene

encoding the membrane-bound protein, *GP130* (92). The factors inducing sgp130 production through alternative splicing are unknown. We have recently shown that about 26 genetic variants contribute to determine sgp130 circulating levels in serum and explain 11% of the variance (93).

Three different sgp130 isoforms have been detected: the full length sgp130, sgp130-RAPS (rheumatoid arthritis [RA] associated peptide) and sgp130-E10 (94-96). While sgp130-RAPS is a 50 kilo Dalton (kDa) molecule, the other two isoforms are 90-110 kDa in size. The sgp130-RAPS was originally discovered in RA patients and is the only isoform verified by Western blot (94, 95). The sgp130-E10 isoform on the other hand accounts merely for 1-2% of circulating sgp130 (95). The isoform most efficient in inhibiting IL6 trans-signalling is the full length sgp130 with inhibition executed in an autocrine fashion in cells secreting protective sgp130 (92, 93). In addition, in an *in vitro* experimental setting, it was demonstrated that monocytes expressed the full-length sgp130 albeit the expression disappeared upon their differentiation into macrophages (92).

1.3.4.3 The interleukin 6 trans-signalling buffer

The pro-inflammatory IL6 trans-signalling pathway contains an intrinsic buffer that prevents an uncontrolled systemic inflammatory reaction driven by the active IL6:sIL6R.

As shown in **Table 1**, a concentration gradient exists among the three circulating components of the IL6 trans-signalling with increasing concentrations from IL6, sIL6R to sgp130 (96, 97). In mild/chronic inflammatory states, IL6 can increase its concentration up to 150 ng/mL while it can further increase a million-fold, exceeding sgp130 levels with potential systemic IL6 trans-signalling, during systemic inflammation/infections such as sepsis (98, 99). The soluble receptor concentrations on the other hand do not change as much and will normally not increase more than 2-5 fold during inflammation (99). Thus, under physiological circumstances sIL6R, being in excessive concentration, will bind the majority of the circulating IL6 (75, 100). Likewise, sgp130 circulates in excess in relation to IL6:sIL6R and this concentration gradient together with the high affinity for sgp130, leads to a rapid formation of a ternary inactive complex (90) (**Table 1**). Being the antagonist of IL6 trans-signalling one would expect sgp130 to play a leading role in the buffer system, albeit sgp130 is incapable of inhibiting IL6 trans-signalling without sIL6R. Hence, the capacity of the buffer is decided rather by the amount of sIL6R than sgp130 as the latter is always in molar excess of sIL6R, and IL6 only in extreme conditions can outnumber both (54, 98). The swift formation of the ternary IL6:sIL6R:sgp130 complex triggered by the sgp130 concentration gradient in the presence of a binary IL6:sIL6R complex leads to the subsequent formation of new binary complexes maintaining the balance between free and bound IL6 (75).

Increasing the sgp130 concentration beyond the physiological concentration gradient will speed up the elimination of binary complexes through ternary complex formation and subsequently drive the formation of new binary complexes hence decreasing the amount of free IL6. At very high concentrations of sgp130, classical signalling can be blocked due to lack of unbound IL6 (75, 90, 92). In case of a simultaneous increase in sIL6R concentration, the depletion of free IL6 will accelerate even further demonstrating how increasing sIL6R concentrations potentiate the buffer (100, 101). In physiological conditions, there will however always exist a small proportion free IL6 enabling classical and trans-signalling to occur simultaneously (75, 101).

1.4 Interleukin 6 trans-signalling in inflammatory diseases

IL6 trans-signalling mediates the transition from acute to chronic inflammation. Signs of active IL6 trans-signalling is demonstrated in several chronic inflammatory conditions such as inflammatory bowel disease (IBD) (102, 103), RA (104, 105), glomerulonephritis (106), cancer (107-113), metabolic syndrome (114) and periodontitis (115). In RA, high levels of sIL6R have been associated with a more severe disease and a monoclonal IL6R antibody, tocilizumab is approved for patients not responding to standard treatment (116, 117). The efficacy of treatment with tocilizumab in RA indicates that the IL6 system plays an important role in the progression of this chronic inflammatory disorder (118). Treatment with tocilizumab affects both the IL6 classical and IL6 trans-signalling inferring side effects from immune system inhibition with increased incidence of infections (119). In addition, increased levels of lipids are seen with tocilizumab treatment. This is however, a sign of successful dampening of chronic inflammation as cholesterol is used as fuel in the energy consuming inflammatory processes and thus lipids are known to be lower in patients with chronic inflammatory conditions (120). It has not been elucidated if this change in cholesterol levels has a pro-atherogenic effect. On the other side, the risk of CVD is reduced in the presence of an effective anti-inflammatory treatment (121). For instance, it was demonstrated in women with RA that treatment with tocilizumab improved two subclinical measures of atherosclerosis; the endothelial dependent vasorelaxation and aortic stiffness (122).

In the search for an immunomodulatory agent to target IL6 trans-signalling, a recombinant form of the antagonist sgp130, sgp130Fc was developed (90). The anti-inflammatory effect of sgp130 is supported by experimental data: in the air-pouch model, using transgenic mice overexpressing sgp130Fc, IL6 trans-signalling was effectively inhibited as was the recruitment of mononuclear cells (123). The sgp130Fc is currently tested in phase II clinical trial of IBD (124).

1.5 Interleukin 6 trans-signalling in atherosclerosis

IL6 is mainly synthesised by endothelial cells and SMC in the vascular wall (125). In **Figure 4,** a few key events of the inflammatory process in atherosclerosis and the role of IL6 trans-signalling are depicted. IL6 trans-signalling participates in the transition from acute to chronic inflammation in the vascular wall and contributes to a sustained inflammatory reaction.

The inflammatory response in atherosclerosis starts with the endothelial cell activation and subsequent loss of the endothelial barrier, secretion of pro-inflammatory interleukins, expression of adhesion molecules and progression towards a prothrombotic state (126). These initial steps are governed by the cells of the innate immune system, monocyte recruitment being one of the earliest features (9, 127). The eliciting factors of atherosclerosis are manifold albeit one of the most central events is the binding and retention of LDL by proteoglycans in the sub-endothelial space (128). In this hyperlipidaemic setting, monocytes are transformed into a more pro-inflammatory cell type secreting pro-inflammatory cytokines such as IL1 and IL6 (127). In an *in vitro* co-culture study, interaction between vascular SMC and monocytes, by means of the pro-inflammatory cytokines IL1, TNF-α and IL6, induced production and secretion of IL6 and monocyte chemoattractant protein 1 (MCP-1) (129).

Taking these findings into the vascular wall it could be speculated that migrating monocytes coming into contact with resident vascular SMC result in increased IL6 and MCP-1 levels further attracting monocyte to the area and stimulating the continued inflammatory process. Monocytes eventually migrate through the disrupted endothelial layer into the sub-intimal space where they are transformed into macrophages stimulated by macrophage colony stimulating factor. Modified by lipases and proteases, ensnared LDL molecules aggregate, are increasingly bound by the surrounding proteoglycans and are then further modified by factors such as reactive oxygen species resulting in the development of oxidised LDL (130, 131). Macrophages engulf the oxidised LDL and are transformed into foam cells expressing scavenger receptors on the cell membrane. SMC migrated from the media into the sub-intimal space can also give rise to foam cells after proliferation and together with macrophage derived foam cells they accumulate in the atherosclerotic plaque (129, 132). Foam cells in the intima die through apoptosis releasing cholesterol crystals forming the central necrotic core of the atherosclerotic plaque. The leakage of cholesterol crystals is one of the ignitors for the IL1β-IL6-CRP pathway as previously described.

The sIL6R is mainly produced by proteolytic cleavage from the cell membrane of local neutrophils stimulated by IL8 and other inflammation associated factors hence increasing concentrations of sIL6R and the assembly of binary IL6:sIL6R complexes (79, 80). IL6 trans-signalling driven by the binary complex enhances

MCP-1 and IL8 secretion from inflamed vascular endothelium thereby promoting a neutrophil – monocyte shift and further augmenting IL6 trans-signalling by stimulating continued shedding of sIL6R and increased IL6 production (79, 129, 133, 134). Moreover, IL6 trans-signalling activates vascular SMC and via up-regulation of their cell surface gp130 expression subsequently enhances IL6 secretion constituting a positive feedback loop resulting in MCP-1 secretion and SMC proliferation (135-137).

Figure 4. Schematic representation of key events in the inflammatory response in atherosclerosis and the role of IL6 trans-signalling

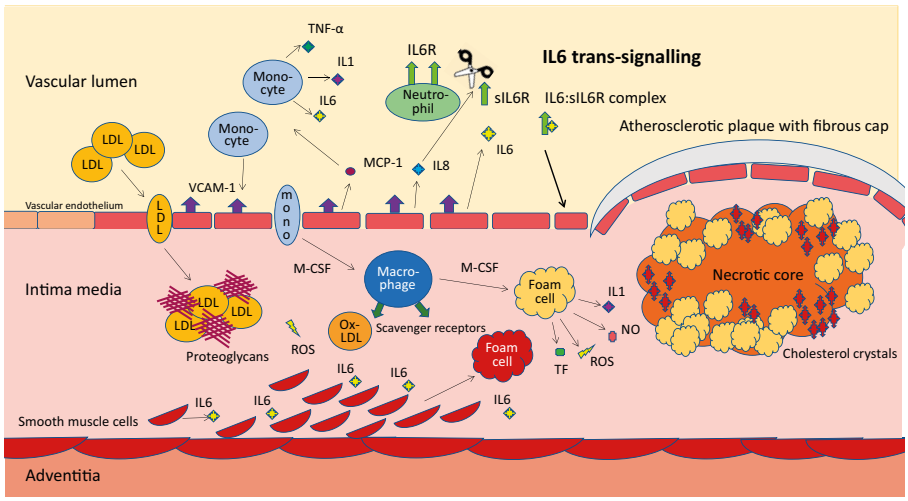

Figure 4. *LDL= low-density lipoprotein cholesterol, VCAM-1=Vascular cell adhesion protein 1, TNF-α=tumour necrosis factor-α, IL=interleukin, MCP-1=monocyte chemoattractant protein 1, Mono=monocyte, IL6R=interleukin 6 receptor, sIL6R=soluble interleukin 6 receptor, M-CSF=macrophage colony stimulating factor ROS=reactive oxygen species, Ox-LDL=oxidised low-density lipoprotein cholesterol, TF=tissue factor, NO=nitric oxide.*

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In addition, thrombin-activated endothelium has been shown to drive the IL6 trans-signalling neutrophil-monocyte transition *in vitro* (79). Apart from demonstrating one potential mechanism behind IL6 trans-signalling in atherogenesis, this study also provided a possible link between inflammation and thrombosis in the interplay between thrombin, IL6 trans-signalling and chronic inflammation in the vessel wall.

Analysing the effects of IL6 trans-signalling in experimental murine models of atherosclerosis, injecting recombinant IL6 resulted in enhanced fatty lesions after 6-21 weeks in one model, while inhibiting IL6 trans-signalling by injecting recombinant sgp130Fc dampened progression of aortic plaques and to some extent even

induced regression of plaques in another (138, 139). More recently in abdominal aortic aneurysm murine models, injecting sgp130Fc increased survival and reduced aortic ruptures, while treatment with an IL6R antibody (blocking both IL6 classical and trans-signalling) had no effect on aneurysm rupture (140).

1.6 Interleukin 6 trans-signalling in coronary artery disease

IL6R is the only gene regulating inflammation whose genetic variants have been causally associated with CAD risk. In a genome-wide association study (GWAS) designed to investigate the association of genetic loci with the risk of CAD, an *IL6R* variant (rs4537545-CT) was associated with increased CAD risk and in addition elevated CRP levels (33). This finding was strengthened by the results from two meta-analyses demonstrating that another *IL6R* variant (rs7529229), in strong linkage disequilibrium with the variant increasing the shedding rate of the IL6R, was associated with higher serum levels of sIL6R and with a decreased risk of CAD (141, 142). Further verification of the association was presented in a GWAS designed to identify novel loci associated with CAD risk and the *IL6R* was one of the 46 loci reaching genome-wide significance which together explain around 10% of CAD heritability (143). Of importance, the direct association between sIL6R levels and CAD risk was not analysed in the above-mentioned studies. Instead, to explain the association of rs7529229 with increased sIL6R levels and reduced CAD risk, the results were compared to that of tocilizumab treatment. The interpretation from the authors was that the lower CAD risk in the presence of high sIL6R levels could be explained by the effect of the IL6R shedding mutation: an increased cleavage of the membrane-bound IL6R caused a decreased IL6R expression and a reduced IL6 classical signalling as observed with tocilizumab treatment (144). Notwithstanding, the finding that the same genetic variant was associated with both increased sIL6R levels and decreased CAD risk is not consistent with the evidence from experimental studies discussed so far and is not in line with the current understanding that IL6 trans-signalling, mainly regulated translationally and post-translationally, is the pathway driving IL6 associated CVD risk. One possible explanation is instead that higher levels of sIL6R potentiate the IL6 trans-signalling buffer system as sIL6R is the key for the antagonist sgp130 to incapacitate the IL6:sIL6R complex (145, 146). Thus, the increased levels of sIL6R are not per se associated with a decreased risk of CAD, but possibly with increased circulating levels of the inactive ternary (IL6:sIL6R:sgp130) complex which in turn might have been associated with a decreased risk of CAD in the meta-analyses discussed.

Several clinical studies have demonstrated the value of IL6 as risk predictor of cardiovascular risk (147). Analysing the association of the soluble IL6 receptors with the risk of CAD, studies on sIL6R are relatively unanimous in showing detrimental effects associated with high sIL6R levels while studies on sgp130 have on the other side demonstrated controversial findings.

High sIL6R levels have been observed in the acute phase of MI (148, 149). Moreover, increased circulating sIL6R levels have been associated with the severity of myocardial injury, with a reduced myocardial reperfusion after percutaneous coronary intervention and with an increased risk of all cause and cardiovascular mortality in patients with ST-elevation MI (148, 150, 151). In all of these studies, sIL6R blood samples were collected in the acute phase of the CVE thus mirroring an acute inflammatory state. In an attempt to investigate the association between sIL6R and CVE in a steady state condition, we measured sIL6R and sgp130 levels three months after the event in a population-based MI case control study with the result that high sIL6R levels were associated with an increased risk of MI while high sgp130 levels seemed to be able to counteract this detrimental effect (152).

On the contrary, circulating levels of sgp130 have been associated with CAD and mortality albeit with controversial results. In patients with stable CAD, plasma levels of IL6 were increased and sgp130 levels decreased while sIL6R did not differ compared to healthy controls (153). In addition, sgp130 levels were negatively correlated with the number of diseased coronary arteries in cases. Similarly, patients with stable or unstable CAD undergoing coronary angiography displayed significantly lower sgp130 levels compared to healthy controls (138). In the same study, patients with unstable CAD and multivessel disease had even lower sgp130 plasma levels in relation to single-vessel diseased and in all unstable CAD patients there was a pattern of augmented IL6 and reduced sIL6R levels. High levels of sgp130 were also associated with increased cardiac morbidity and mortality in elderly patients with heart failure of ischemic origin (154). Overall there is a controversy between the data on sgp130 from the experimental and observational studies.

One possible explanation is that it is hard to interpret the impact of sgp130 as well as the association of IL6R with the CAD risk on its own as the entire signalling system relies on the relative concentrations of the three biomarkers. In particular, the function of sgp130 is tightly dependent on the presence, availability and concentration of the binary IL6:sIL6R complex.

1.7 Interleukin 6 trans-signalling in cerebrovascular disease

Ischemic cerebrovascular disease consists of a group of conditions with similar neurological symptoms but with a plethora of underlying pathophysiological processes. Categorising ischemic stroke subtypes based on aetiology, the Trial of Org 10172 in Acute Stroke Treatment (TOAST) defined subgroups of ischemic stroke: large vessel disease, small vessel disease, cardioembolism, stroke of other determined aetiology and stroke of undetermined aetiology (155). In this thesis, the focus will be on the association of IL6 trans-signalling with large vessel cerebrovascular diseases and with cardioembolism.

1.7.1 Interleukin 6 trans-signalling in large vessel cerebrovascular disease

The function of IL6 signalling in the ischemic brain is not entirely evident as conflicting results with both detrimental pro-inflammatory and regenerative and anti-inflammatory effects have been demonstrated in experimental and clinical studies (156).

In experimental models, increased IL6 levels are induced by injury to the carotid artery (157). Similarly, acute cerebrovascular occlusion is associated with elevated levels of IL6 in clinical studies (158-160). In addition, high serum IL6 associates with asymptomatic carotid artery stenosis and signs of unstable carotid plaques (161-164). Within the first days after an ischemic stroke, circulating IL6 increases and correlates with infarct size, early neurologic deterioration and long-term poor outcome (156, 158). Furthermore, expression of IL6 in neurons, astrocytes and glia is enhanced in the sub-acute phase of stroke (159, 165, 166). In the acute phase of cerebral ischemia, IL6 expression and secretion is increased with elevated concentrations found in the cerebrospinal fluid greater than circulating serum levels, indicating local IL6 production in the central nervous system (CNS) (159, 167). In an experimental model of transient middle cerebral artery occlusion in rats, expression of IL6 was concentrated to neurons in the cerebral cortex in areas surrounding the infarction from 3 hours up to 7 days after the exposure (165).

Investigating the ambivalent properties of IL6 in CNS, a neuroprotective effect was demonstrated when injecting recombinant IL6 into the cerebral ventricles of rats (166, 168). However, no difference in the infarct size or neurologic function between IL6 knockout mice and wildtype littermates was seen 24 h after induced cerebral ischemia (169).

Current knowledge of the effect of IL6 signalling proposes that IL6 classical signalling is neuroprotective (170, 171). Exploring the effects of the IL6 trans-signalling pathway in relation to classical IL6 signalling in the CNS, the distribution of IL6R is crucial. *IL6R* mRNA has been quantified in microglia, astrocytes and neurons albeit the IL6R protein has solely been detected in microglia (172). As microglia express the IL6R it can be subject to classical IL6 signalling whereas neurons and astrocytes only respond to the binary IL6:sIL6R complex indicating that they lack the IL6R, the membrane-bound receptor (173, 174).

IL6 trans-signalling is proposed to be responsible for neuronal degeneration in cerebral ischemia. In a transgenic murine model with astrocytes overexpressing IL6, localised neuroinflammation and secondary neuronal deficits were demonstrated (175). In an attempt to explore IL6 trans-signalling as the cause of neuroinflammation the same research group developed a bigenic mouse with the same CNS-restricted production of IL6 but with the addition of localised sgp130Fc production and showed mitigated effects of inflammation (176). Moreover, in a model of lipopolysaccharide-induced neuroinflammation, intraventricular injections of sgp130 accelerated the recovery in mice (177).

Clinical studies on IL6 trans-signalling and ischemic stroke are extremely scarce. Research on sIL6R is inconsistent, with two case control studies displaying no difference in sIL6R levels between ischemic stroke cases and controls whereas levels of IL6 were higher in cases (160, 178). Moreover, in subjects with manifest CVD, sIL6R showed no association with recurrent CVE while in post-MI patients, high circulating levels of sIL6R were associated with increased risk of recurrent CVE (151, 179). Levels of sgp130 have been demonstrated to be transiently reduced after stroke and to correlate positively with blood pressure and carotid intima media thickness in stroke patients (160, 180).

The current understanding is thus that classical IL6 signalling has a regenerative role in the CNS while neuronal degeneration is governed by IL6 trans-signalling.

1.7.2 Interleukin 6 trans-signalling in cardioembolic cerebrovascular disease

AF is a common arrhythmia associated with an increased risk of ischemic stroke (181). The exact mechanism is not fully understood but the current understanding is based on the interaction of several mechanisms such as those in Virchow's triad (182, 183). From the research underlying the CHADS-VASc score, the most widely used risk score to evaluate stroke risk in AF, it is known that certain factors such as age, sex, hypertension, diabetes mellitus, heart failure, prior ischemic cerebral

event, prevalent CVD, increase the risk of thromboembolism in AF (184). In spite of the prothrombotic and pro-inflammatory state described in AF, scores such as the CHADS-VASc do not take components of inflammatory pathways or coagulation cascade into account (185). The association between circulating IL6 levels and risk of AF and secondary thromboembolic events has been investigated in a handful of studies describing increased levels of IL6 in AF patients compared to healthy controls (185-187). IL6 levels are positively correlated with the presence and duration of AF in addition to the diameter of the left atrium (188). Furthermore, multiple studies have demonstrated an association between IL6 serum levels with the occurrence of AF after cardiac surgery/intervention (186, 189-192). Increased levels of IL6 have also been associated with a prothrombotic state and increased risk of thromboembolic events in AF patients (186). On the other hand, in a case control study, patients with AF had high IL6 levels but without an association with markers of endothelial activation or active thrombogenesis and treatment with oral anticoagulant therapy left IL6 levels unchanged (193). IL6 in general has been quite thoroughly studied in relation to AF, thromboembolism and stroke, while the IL6 trans-signalling pathway specifically has not.

In two Mendelian randomisation studies analysing the association of the *IL6R* with AF and secondary ischemic stroke, a causal association was demonstrated (194, 195). In addition, the more recent of the two studies proposed that the increased risk of cardioembolic stroke associated with the *IL6R* was accounted for by AF (195).

2 AIMS OF THE THESIS

The overarching aim of this thesis is to analyse the association of the circulating IL6 trans-signalling, estimated by a ratio between the active binary IL6:sIL6R complex and the inactive ternary IL6:sIL6R:sgp130 complex (the binary/ternary complex ratio [B/T ratio]), with the risk of CVE and to shed a light on the role of IL6 trans-signalling in clinically manifest atherosclerosis.

Aim of each study within the thesis:

Study I To analyse the association between the B/T ratio and the risk of future CVE in a population-based study of 60-year-old men and women from Stockholm.

Study II To analyse the association between the B/T ratio and the risk of future CVE in individuals at low-intermediate risk of CVE.

Study III To investigate if IL6 and the membrane-bound and soluble IL6R and gp130 genes are expressed in carotid artery plaques of patients undergoing carotid endarterectomy due to carotid artery stenosis.

Study IV To analyse the association between the B/T ratio and the risk of future ischemic stroke in relation to atrial fibrillation.

3 METHODS

3.1 The cohort of 60-year-old men and women from Stockholm

3.1.1 Study population in Study I, II, IV

Results from Study I, II and IV were derived from the cohort of 60-year-old men and women from Stockholm (60YO). The 60YO is a prospective population-based cohort designed to identify novel cardiovascular risk factors and biomarkers. From August 1997 to March 1999, every third man and woman born between July 1st 1937 and June 30th 1938 residing in the Stockholm County were randomly selected from the Swedish Population Register (n=5460, 2779 men and 2681 women) and received a letter of invitation to participate in a cardiovascular health screening study. The letter contained a complete description of the study and stated clearly that participation was voluntary. In addition, recipients were informed that results from the health screening would be interpreted by a physician and in case of pathological findings participants would be given appropriate medical advice. Recipients were asked to contact a study nurse to state whether they were interested in participating or not. Participants who accepted to enter the study gave the study nurse their oral consent which was then recorded in the study case report form. Written consent forms were not mandatory in Sweden at the initiation of the study. Each reply was documented and those who accepted participation received an appointment for the health screening and were sent the comprehensive study questionnaire while those who were not interested were withdrawn from the list of eligible study participants. With a 77.5% positive response rate, 4232 participants were included in the study (2193 women and 2039 men). At the baseline visit, participants met with a study nurse and underwent a physical examination including height and weight measurements and blood pressure measured after 5 min of rest in a sitting position using an automatic device (HEM 711; Omron Health Care, Bannockburn, IL) with the mean of two values calculated. A wider cuff was used if the participant had an upper arm >32 cm in circumference. Each participant had an electrocardiogram (ECG) recorded and blood samples were drawn after overnight fasting from an antecubital vein. Plasma, serum and whole blood were separated, immediately frozen at −80 °C and stored in a biobank. Certain biochemical tests were run in parallel with the screening including fasting levels of serum lipids analysed with enzymatic methods (Bayer Diagnostics, Tarrytown, NY, USA) and serum glucose measured with an enzymatic colorimetric test (Bayer Diagnostics). LDL cholesterol was estimated using the Friedewald method.

Demographic data as well as participants' anamnestic records on lifestyle habits, details on current and previous diseases and medication were obtained from the self-administered questionnaire sent home to participants upon acceptance to participate. The answers in the questionnaire were checked together with the study doctor at the baseline visit.

For all analyses presented in this thesis, participants with an incomplete study questionnaire (n=122), prior CVD i.e. either self-reported or diagnosis code of CVD in the national registers before enrolment (n=369) or lacking serum samples for the measurement of IL6, sIL6R and sgp130 (n=96) were excluded, leaving 3645 study participants as seen in the flow chart in **Figure 5**.

Figure 5. Flow chart of the inclusion/exclusion criteria in the cohort of 60-year-old men and women from Stockholm adopted in Studies I, II and IV

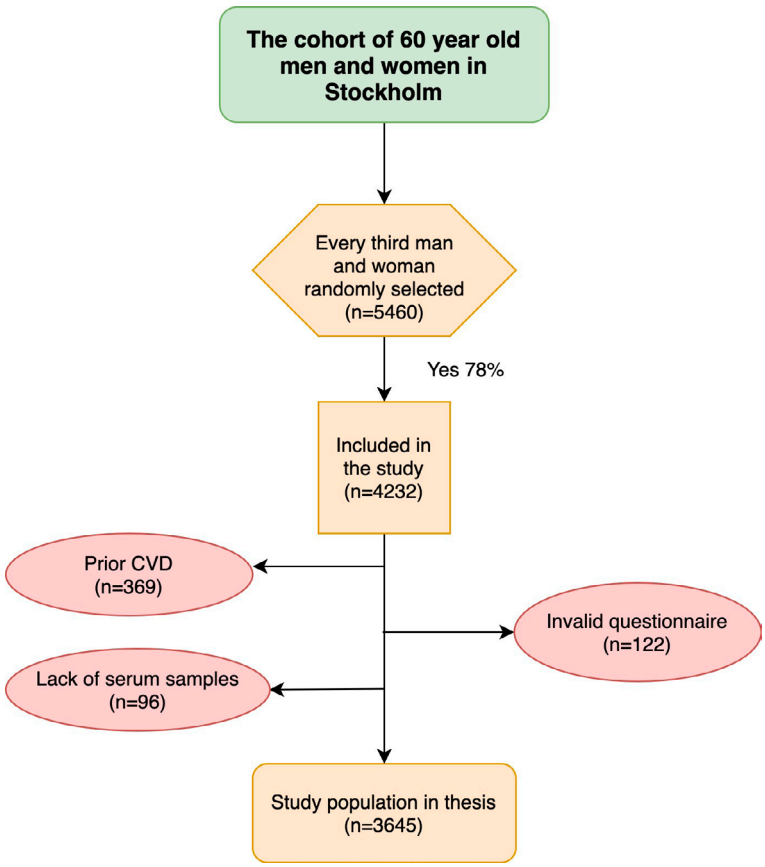


Figure 5. *Ziegler 2020 (unpublished)*

3.1.2 Outcome ascertainment in Study I, II, IV

To extract the outcome diagnoses, the personal identification number of each study participant was linked on a regular basis to the mandatory national Swedish registers; The Swedish National Inpatient Register and The National Cause of Death Register. The Swedish National Inpatient Register started in 1964 and reached complete (99%) national coverage of all somatic and psychiatric hospital discharge diagnoses in Sweden in 1987 with a positive predictive value on diagnoses from this register of 85-95% on average (196). The Inpatient Register does not contain diagnoses from primary care. The National Cause of Death Register with national coverage since 1952 contains date of death and associated primary cause of death with accompanying diagnoses recorded on all deceased in Sweden and Swedes deceased abroad (197).

In Study I and II, the primary endpoint was first time incident fatal and non-fatal composite CVE recorded until December 31st 2014 in the national registers. Subjects with the following diagnosis codes according to the International Classification of Diseases 10th revision (ICD-10) were categorised as a case: I21 MI; I20 and I25 coronary heart disease; I46 cardiac arrest with/without successful resuscitation; and I63 ischaemic stroke. Fatal CVE was defined as sudden cardiac death or death with the underlying diagnosis codes I21 (MI) or I63 (ischaemic stroke). Solely main diagnoses were recorded. After exclusions had been applied, the study population consisted of 546 incident cases of CVE until December 31st 2014. From the study population, 21 subjects were additionally excluded since they had been inaccurately classified as cases with one of the following ICD-10 diagnosis codes: I259 chronic ischemic heart disease (n=4) as this implies prevalent CAD; I252 old MI (n=1) as this diagnosis similarly implies manifest CAD or at least introduces uncertainty of the exact time of the first presentation of CAD; I652 carotid artery stenosis (n=8) as this diagnosis not necessarily entails an ischemic stroke; and I649 stroke, not specified as haemorrhage or infarction (n=8) since the nature of the cerebrovascular event could not be ascertained. After the final exclusions had been executed, 525 cases of CVE remained in the data set for analyses.

In Study II, the secondary endpoints were either fatal or non-fatal ischaemic stroke (ICD-10: I63) or coronary event (ICD-10: I20, I21, I25, I46), respectively. In the analysis of incident ischaemic stroke, cases of incident CAD events (n=361) were excluded and similarly cases of ischaemic stroke (n=164) were excluded when analysing the risk of incident CAD.

In Study IV, the primary endpoint was fatal and non-fatal ischeamic stroke (I63). In this study, cases of CVE until December 31st 2017 had been ascertained in national registers. Using the same exclusion and inclusion criteria as in Study I

and II, the study population consisted of 655 incident CVE. In line with exclusions performed in secondary analyses in Study II, individuals with an incident CAD event during follow-up were excluded (n=433). In addition, subjects inaccurately categorised as incident ischemic stroke with ICD-10 diagnosis codes I649 (stroke, not specified as haemorrhage or infarction) or I652 (carotid artery stenosis) were excluded (n=19). The total number of incident ischemic strokes after inaccurate diagnoses had been excluded was 203.

Incident AF was the secondary endpoint in Study IV. Both main and secondary diagnoses of AF (ICD-10: I48) were ascertained from registers. To estimate the association of IL6 trans-signalling with the risk of AF, individuals with prevalent AF at baseline (n=29) were excluded i.e. those with either self-reported AF, AF on the baseline ECG or the ICD-10 diagnosis code I48 in national registers before inclusion. In addition, cases of incident ischemic stroke (n=203) were excluded. Given that the B/T ratio was associated with the risk of incident CVE in Study I-II (**Figure 11, Table 4**), keeping incident cases of ischemic stroke in the referent group might have attenuated a potential association with AF.

3.1.3 Biomarker measurement in Study I, II, III and IV

The IL6, sIL6R and sgp130 were measured in serum in Study I, II and IV and in plasma in Study III.

Serum and plasma concentrations of IL6 and sIL6R were measured with Meso Scale Discovery Human Cytokine Assay (Gaithersburg, MD, USA). To measure IL6, samples were diluted 1:2 and to measure sIL6R diluted 1:50. Concentrations were derived from an experimental standard curve and expressed in pg/mL (IL6) and ng/mL (sIL6R). The lower limit of detection for IL6 was 0.06 pg/mL and for sIL6R 0.1 ng/mL.

Serum and plasma sgp130 was measured by means of an assay development kit (#DY228) provided by R&D Systems (R&D systems Minneapolis, MN, USA) and samples were diluted 1:100. The experiment was performed in 96-well plates coated with diluted capture antibody (4.0 lg/mL) incubated overnight at room temperature. After blocking further binding with 2% bovine serum albumin the diluted serum/plasma sample was added. After 2 hours incubation, detection antibody (0.08 lg/mL), Streptavidin and substrate colour reagent were added stepwise. Ultimately, the plate was read in a microplate reader (Tecan, Infinite F200) set to a wavelength of 450 nm. Concentrations of sgp130 expressed in ng/mL were derived from the standard curve. A recombinant sgp130 with a known concentration of 5 ng/mL was used to validate the concentrations estimated in the experiment. The lower limit of detection for sgp130 in our experimental condition was 1.69 ng/mL.

All measurements were performed according to the protocol from the assay manufacturer and the researcher setting up the plate templates for all experiments was blind to study participants' case/referent status.

The variability of concentration values within an assay, the intra-assay variability and the variability of concentration values between assays, the inter-assay variability were calculated with the following formula: Coefficient of variation = duplicate standard deviation/duplicate mean.

The intra-assay variability was 6.3% for IL6 with 478 duplicates, 4.7% for sIL6R with 284 duplicates, and 4.5% for sgp130 with 395 duplicates. The data presented represent the average of the coefficients of variation of duplicate samples run in the same assay.

The inter-assay variability was 3.5% for IL6, 7.5% for sIL6R, and 6.0% for sgp130 and represents the average of the coefficient of variation of duplicate samples analysed in 32 sequential plates using the same formula as for intra-assay variability presented above.

The recommended threshold is 15% for mean intra-assay coefficient of variation and 18% for mean inter-assay variation for IL6 and sIL6R according to the manufacturer. No limits for intra- and inter-assay variability were suggested by the manufacturer for sgp130, albeit in prior studies an intra-assay variability <10–11% and inter-assay variability <10–16% have been reported (198, 199).

3.1.4 Calculating the binary and ternary IL6 complexes in Study I, II, IV

The constituents of the IL6 complexes interact on a molar basis in the circulation. The molar (M) concentration, i.e. moles per litre of IL6, sIL6R, and sgp130 for each individual was calculated by dividing the serum concentration (ng/mL) by their respective molecular weights (IL6 23.7 kDa, sIL6R 50 kDa, and sgp130 100 kDa). The nanomolar (nM) concentration of the binary and ternary complex was then estimated using the formulas presented by Müller-Newen et al. (75, 101).

$$[\mathbf{IL6}:\mathbf{sIL6R}]=0.5[\text{sIL6R}]i+0.5[\text{IL6}]i+0.5K_{D1}-0.5([\text{sIL6R}]i^2+[\text{IL6}]i^2+2[\text{IL6}]iK_{D1}+K_{D1}{}^2)^{0.5}$$

$$[\mathbf{IL6}:\mathbf{sIL6R}:\mathbf{sgp130}]=0.5[\text{sgp130}]i+0.5[\text{IL6:sIL6R}]i+0.5K_{D2}-0.5([\text{sgp130}]i^2+[\text{IL6:sIL6R}]i^2+2[\text{IL6:sIL6R}]iK_{D2}+K_{D2}{}^2)^{0.5}$$

The [IL6]i, [sIL6R]i, and [sgp130]i were substituted with their respective nM serum concentrations. The dissociation constant values; binary complex $K_{D1}=0.5$ and ternary complex $K_{D2}=0.05$ nM, were deducted from the original analysis of Müller-Newen and colleagues (75). To ascertain that the association between the IL6 complexes and the outcome would be valid in case of the dissociation constants

used being inadequate, the relative amount of the binary and ternary complexes replacing K_{D1} and K_{D2} with values ten times higher and ten times lower was calculated using the same formula.

3.1.5 Ethics in Study I, II, IV

The 60YO was designed and conducted in accordance with the Helsinki Declaration. The study, including the procedure with oral consent documented by the study nurse, was approved by the Regional Ethics Review Board at Karolinska Institutet in Stockholm in the original application in 1996 (reference number 96-398) and in renewed applications addressing continued and extended research (reference numbers 99-306, 03-100 and 03-115, 16/205-31/2).

3.2 The Biobank of Karolinska Endarterectomies

3.2.1 Study population in Study III

Results from Study III were derived from the Biobank of Karolinska Endarterectomies (BiKE) cohort. The BiKE study, is a real-world clinical study analysing large vessel cerebrovascular disease by including patients amenable for carotid endarterectomy (CEA) due to a high-grade carotid artery stenosis according to the North American Symptomatic Carotid Endarterectomy Trial (NASCET) criteria (200). Patients about to undergo CEA at the Department of Vascular Surgery, Karolinska University Hospital or the Section for Vascular Surgery at the Department of Surgery, Södersjukhuset Hospital, Stockholm, Sweden were sequentially enrolled in the BiKE study in 2002-2007. Exclusion criteria were: extensive neurological deficits or large cerebral infarcts as the risk of surgery would outweigh the benefits and diagnosis of atrial fibrillation since this diagnosis possibly implicates a cardioembolic source of embolism rather than from the carotid artery.

Patient clinical data, including history of diseases, chronic diagnoses, ongoing medical treatment, life style habits, were obtained on admission to hospital. Blood samples and carotid plaques were amassed at surgery. Under local anaesthesia CEA was performed and the plaque retrieved cut in half in the most stenotic region. The proximal portion of the plaque was directly frozen at −80 °C and kept in the BiKE study biobank together with the blood samples.

The analysis performed in this thesis was analysed in a plaque profiling subset (n=97) of the BiKE cohort (201). In the flow chart in **Figure 6**, the exclusions performed in the present analysis of the BiKE study are shown. Participants were excluded from analyses due to incomplete clinical data (n=4), lack of samples for the semi-quantitative real time polymerase chain reaction (semi-qRT-PCR) (n=11) or a standard deviation >0.5 in the semi-qRT-PCR (n=4).

Participants with contralateral cerebral deficit symptoms < 6 months preceding CEA were categorised as symptomatic and those without symptoms of neurological deficit or symptoms ≥ 6 months prior to CEA as asymptomatic. For the latter, CEA indication was based on the ACST (Asymptomatic Carotid Surgery Trial) results (202).

Symptoms of neurological deficit, potentially mirroring plaque instability, were either transient (<24 hours) as in transitory ischemic attack (TIA) or amaurosis fugax (transient retinal ischemia) or permanent as in ischemic stroke. Out of the 78 patients in the present analysis, 53 were defined as symptomatic and 25 as asymptomatic.

Figure 6. Flow chart of study population from a BiKE subset in Study III

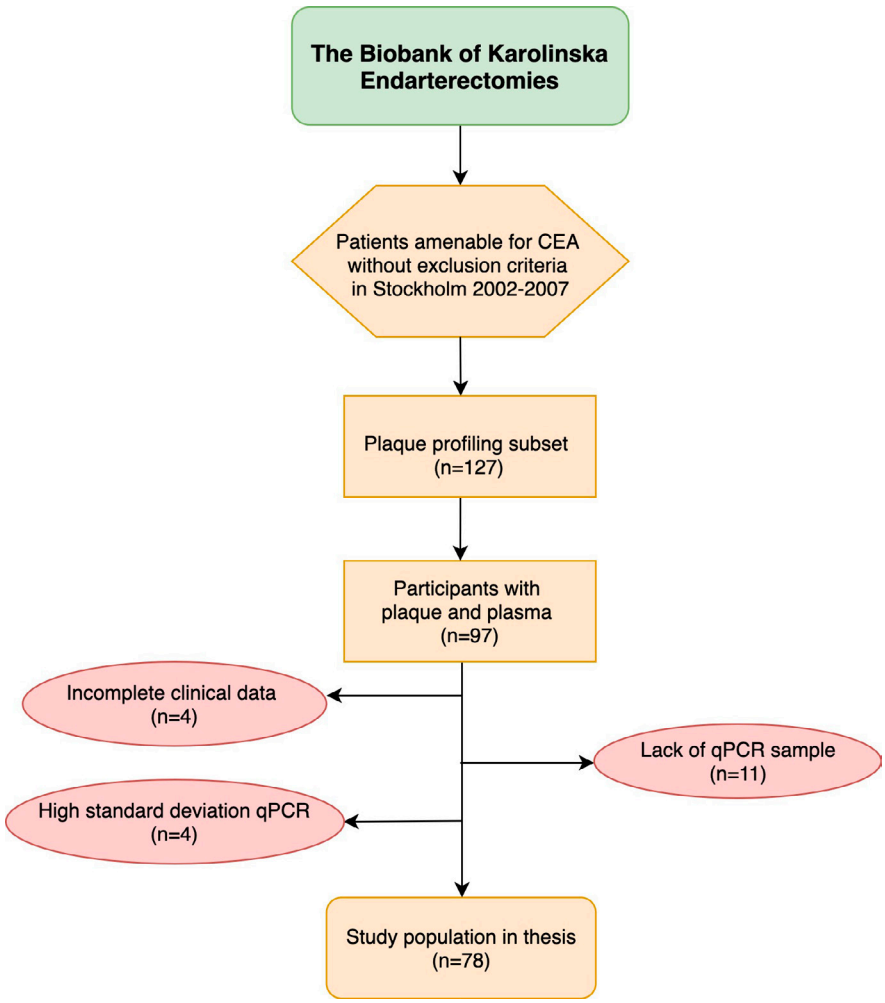


Figure 6. *Ziegler 2020 (unpublished)*

3.2.2 Gene expression analyses in Study III

Gene expression levels of *IL6* had been previously analysed in the same samples and expression (log2) had been estimated by Affymetrix HGU133plus2 microarrays (201). The microarray dataset is available from Gene Expression Omnibus (GSE21545).

3.2.2.1 Oligoprimer design

Oligoprimers were designed to selectively amplify and thereby quantify the expression of *IL6R, sIL6R, GP130* and s*GP130-RAPS* (s*GP130*) in carotid artery plaques by Bruna Gigante (BG) and Jasmine Lundqvist (JL). For the membrane-bound (*IL6R; GP130)* and soluble (*sIL6R;* s*GP130-RAPS)* receptors, oligoprimers were designed to selectively amplify each receptor individually.

The *IL6R* cDNA sequence NM_000565.4 (base pairs [bp] 1261-1560) together with the oligoprimers designed to amplify the two IL6 receptors, *IL6R* and s*IL6R* are presented in **Figure 7**. The s*IL6R* lacks a 94-bp fragment coding the IL6R transmembrane (TM) region as a result of alternative splicing. In the oligoprimer sequences designed to amplify s*IL6R*: the reverse primer spans across the splicing junction, while the IL*6R* reverse primer lies in the TM region.

Figure 7. Schematic representation of the IL6R cDNA and oligoprimer sequences chosen to amplify the IL6R and the sIL6R

1261 ACGAGGTGTC CACC**CCCATG CAGGCACTTA CTACT**AATAA AGACGATGAT AATATTCTCT
1321 TCAGAGATTC TGCAAATGCG ACAAGCC**TCC CAG**TGCAAGA TTCTTCTTCA GTACCACTGC
1381 CCACATTCCT GGTTGCTGGA GGGAGCCTGG CCTTCGGAAC GCTCCTCTGC ATTGCCATTG
1441 TTCTGAG***GTT CAAGA*****AGACG T**GGAAGCTGC GGGCTCTGAA GGAAGGCAAG ACAAGCATGC
1501 ATCCGCCGTA CTCTTTGGGG CAGCTGGTCC CGGAGAGGCC TCGACCCACC CCAGTGCTTG

	Oligoprimer sequences	Amplicon length (bp)
***IL6R* forward**	5'-AGAGATTCTGCAAATGCGACAAG-3'	133
***IL6R* reverse**	5'-TCTTGAACCTCAGAACAATGGC-3'	
***sIL6R* forward**	5'-CCCATGCAGGCACTTACTACT-3'	93
***sIL6R* reverse**	5'-ACGTCTTCTTGAACCTGGGA-3'	

Figure 7. *Oligoprimers used to amplify the membrane-bound IL6R are written in red: the forward (bp 1323-1345) and the reverse primer (bp 1434-1455). The reverse primer lies in the sequence coding the TM domain (underlined).*

Oligoprimers used to amplify the sIL6R are written in bold black: the forward (bp 1274-1294) and the reverse primer (bp 1348-1353 and 1448-1461). The reverse primer crosses the splicing junction and overlaps from bp 1448 to bp 1455 with the IL6R reverse primer (highlighted in bold cursive red).

The oligoprimer sequences and amplicon length are reported in the table under the figure.

In **Figure 8.** *GP130* cDNA sequences are represented schematically.

Panel A shows the nucleotide sequence of the membrane-bound *GP130* isoform 1 (GenBank accession number: NM_002184.4 [bp 1251-1420]) and Panel B shows the soluble *GP130* isoform 2 also known as s*GP130-RAPS* (NM_175767.3 [bp 1201-1420]) (94, 203, 204).

The soluble sgp130-RAPS is produced by alternative splicing of exon 9 (203): The alternative splicing results in a stop codon and creates a shorter protein lacking the TM domain (exon 15) (203). The only sgp130 isoforms verified by Western blot is sgp130-RAPS (95).

Figure 8. Schematic representation of the GP130 cDNA and oligoprimer sequence (Panel A.) and of the sGP130-RAPS cDNA and oligoprimer sequence (Panel B.)

PANEL A.

1251 AAGATAGACC ATCTAAAGCA CCAAGTTTCT GGTATAAAAT **GATCCATCC CATACTCAAG**
1311 **G**CTACAGAAC TGTACAACTC GTGTGGAAGA CATTGCCTCC TTTTGAAGCC AATGGAAAAA
1371 TCTTGGATTA TGAA**GTGACT CTCACAAGAT GGA**AATCACA TTTACAAAAT TACACAGTTA

PANEL B.

1231AGCAAGTGGG ATCACCTATG AAG**ATAACAT TGCCTCCTTT TGAAGCC**AAT GGAAAAATCT
1291 TGGATTATGA AGTGACTCTC ACAAGATGGA AATCACATTT ACAAAATTAC ACAGTTAATG
1351 CCACAAAACT GACAGTAAAT **CTCACAAATG ATCGCTATCT AGC**AACCCTA

	Oligoprimer sequences	Amplicon length (bp)
***GP130* forward**	5'-GATCCATCCCATACTCAAGG-3'	112
***GP130* reverse**	5'-TCCATCTTGTGAGAGTCAC-3'	
s*GP130* forward	5'-ATAACATTGCCTCCTTTTGAAGCC-3'	140
s*GP130* reverse	5'-GCTAGATAGCGATCATTTGTGAG-3'	

Figure 8. Panel A*: Oligoprimers used to amplify the membrane-bound GP130 are written in bold black: the forward (bp 1291-1311) and reverse primer (bp 1384-1403). The forward oligoprimer lies in the sequence coding exon 9 (underlined) with the donor and acceptor sequences at the splicing junction (underlined in red). Alternative splicing of exon 9 results in the alternative GP130 transcript (reported in Panel B) which translates in the soluble isoform sgp130-RAPS (sGP130).* ***Panel B****: Oligoprimers used to amplify the soluble gp130-RAPS (sGP130) are written in bold black: forward (bp 1254-1276) and reverse (1371-1393) oligoprimers. This isoform lacks exon 9 which is present in the membrane-bound gp130. The forward oligoprimer spans the splicing junction (underlined in red). Oligoprimer sequences and amplicon length are reported in the table under the figure.*

In preliminary experiments we tested four housekeeping genes, *β-ACTIN*, glyceraldehyde 3-phosphate dehydrogenase (*GADPH*), *β2-microglobulin* and *16S ribosomal-RNA*. Two of them *β-ACTIN* and *GAPDH* were chosen since they could be successfully amplified together with the *IL6R*. The *β-ACTIN* oligoprimer was designed on cDNA sequence NM_001101 and the *GADPH* primer on cDNA sequence NM_002046. The oligoprimers were designed based on sequences extracted from GenBank and the sequences are shown in **Table 2.**

Table 2. Oligonucleotide primers for housekeeping genes, *β-ACTIN* and *GAPDH*.

Gene	Primer	Sequence	Amplicon length (bp)
β-ACTIN	Forward	5'-GTGATGGACTCCGGTGACG-3'	191
	Reverse	5'-TTCTCCTTAATGTCACGCACGAT-3'	
GAPDH	Forward	5'-ACCCACTCCTCCACCTTTGAC-3'	100
	Reverse	5'-TGTTGCTGTAGCCAAATTCGTT-3'	

3.2.2.2 Validation of oligoprimers

Preliminary experiments were performed to validate the primer sequences. The cDNA was reverse transcribed by the QuantiTect Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany) from total RNA extracted from HepG2 cells. Polymerase chain reaction (PCR) was then performed for each of the primers set with 0.5 pmol/µl of forward and reverse primers, 0.02 U/µl Phusion DNA polymerase, 1X Phusion HF buffer, 200 pmol/µl dNTP, 0.5 ng/µl template cDNA in a 20 µl reaction. The cycles used were 98 °C for 2 min, followed by 35 cycles at 98 °C for 10 s and 60 °C for 30 s and 72 °C for 30 s. The PCR products were loaded on a 3% agarose gel with GelRed® Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). After gel electrophoresis and UV-examination, fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany). Amplification of PCR products was verified through sequencing at the KIGene core facility (Karolinska Institutet, Sweden).

3.2.2.3 Probe design

TaqMan® probes were custom designed by Applied Biosystems (Thermo Fisher Scientific) to be compatible and selective for our oligoprimer/gene sequences.

IL6 signalling receptors were made as minor groove binder probes and housekeeping genes as QSY probes, so that two minor groove binder probes could be co-amplified with the two housekeeping genes in one multiplex. Applied Biosystems manufactured a designed assay mix (containing primers and probes) for each IL6

receptor and for *β-ACTIN* and *GAPDH*. Applied Biosystems vouch that each assay has passed bioinformatic quality control and is predicted to be specific. Lastly, the assays are not predicted to interact with each other in a multiplex reaction.

As last step in the validation of the primers and probe sequence, cDNA at different concentration (0.2-0.5 ng/microliter) was amplified as singleplex as well as in multiplex in all assay combinations. From these experiments, an optimal oligo-primer/probe concentration was derived. In the multiplex reaction, *IL6R, sIL6R, GP130* and s*GP130-RAPS* were used at a concentration of 900 nM for each primer and mixed with 250 nM of probe while *β-ACTIN* and *GAPDH* were used at a concentration 225 nM for each primer and mixed with 62.5 nM probe.

3.2.2.4 Real time semi-quantitative PCR

Total RNA (3.3 ng/ul) extracted from carotid endarterectomies was reverse transcribed to cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN GmbH, Hilden, Germany) and random primers as described previously (205).

Five nanograms of cDNA was used for each semi-qRT-PCR reaction, which were run in duplicates, using the TaqMan Multiplex Master Mix on an Applied Biosystems 7500 Real-Time PCR system. The cycles used were 95 °C for 20 s, followed by 40 cycles at 95 °C for 3 s and 60 °C for 30 s. The genes were multiplexed in two sets. The first included *IL6R, sIL6R, β-ACTIN* and *GAPDH*, and the second *GP130,* s*GP130-RAPS, β-ACTIN* and *GAPDH*. Oligoprimers/probe concentrations in each reaction are outlined above.

JL and Kristian Dreij (KD), who ran the semi-qRT-PCR, were not aware of the symptomatic and asymptomatic status of the patients included in the study. Comparison of gene expression levels of *IL6R, sIL6R, GP130* and s*GP130*-RAPS was quantified by the comparative cycle threshold (C_T) method (206). The difference between the cycle threshold (C_T) for each target gene and the average C_T of the two housekeeping genes (ΔC_T) was calculated for every sample. Since gene expression is inversely related to the ΔC_T threshold value, the negative ΔC_T value was used in the analyses to facilitate interpretation of the results.

3.2.3 Ethics Study III

The BiKE study followed the Declaration of Helsinki guidelines with human samples and clinical data collected with informed consent from patients. The study was approved by the Ethical Committee of North Stockholm **(**ethical permit numbers: 95-276/277; 01-199; 02-147; 2009/512-31/2).

3.3 Statistical analyses

In all the four studies, continuous variables are presented as median with an interquartile range (IQR) while binary variables are presented as proportion or percentages. For all statistical analyses, the significance threshold for the *p* value was set at ≤0.05.

All statistical analyses were performed in Stata Corp. (Stata statistical software: Release 14. College Station, TX: StataCorp LP) except for Study I in which R (www.r-project.org) was used to analyse the association with natural cubic splines and in Study II, NRI was calculated using SAS version 9.4 (SAS Institutet, Cary, NC, USA).

3.3.1 Study I

To estimate the relative risk of future CVE associated with IL6 trans-signalling, a ratio between the binary and ternary IL6 complexes was calculated based on the estimated molar concentrations of each component as described above.

The risk of CVE associated with the B/T ratio was assessed using Cox proportional hazards models expressing risk as hazard ratio (HR) with a 95% confidence interval (CI). In preliminary analyses, each of the IL6 trans-signalling components; IL6, sIL6R, and sgp130, serum levels were analysed using Cox models. The dependent variable in all analyses was time to incident CVE while IL6, sIL6R, sgp130 serum levels, and the B/T ratio were predictors. Censoring was done after the occurrence of first time CVE, death or at the end of follow-up which ever came first. Schoenfeld's test was performed to test proportionality of hazards.

To test for non-linear associations, the effect of each predictor (IL6, sIL6R, sgp130 and the B/T ratio) on the response variable (time to CVE) was modelled by means of natural cubic splines. Modelling was performed using the statistical software R and the function 'ns' in the R package 'splines'. A spline basis with four internal knots at the empirical quantiles was chosen. As IL6, sIL6R, and the B/T ratio seemed to have a linear association with the time to CVE, they were modelled in a regression equation using a linear function and the CVE risk expressed as HR (95% CI) per unit increase in serum concentration. The association of sgp130 with the risk of CVE on the other hand did not appear to be linear. As the distribution of the B/T ratio in the study population displayed a narrow range of values (IQR 1.55-1.62), the relative risk associated with 0.1 unit increase of the ratio was analysed in the regression model estimating the CVE risk with the B/T ratio expressed as a continuous variable.

As seen in the graph plotted from the natural cubic spline analysis of the B/T ratio as a continuous variable, there was a pattern of lower risk associated with the B/T

ratio <the median (B/T ratio 1.59) and increased risk >median hence the median was chosen as a cut-off the B/T ratio (**Figure 11**). To further test this assumption, the B/T ratio was categorised into three groups with the referent group including the median (40-60th percentiles = B/T ratio 1.57–1.60 under the assumption of K_{D1} = 0.5 nmol/L and K_{D2} = 0.05 nmol/L) and the group with the assumed lowest risk due to a relative excess of the ternary complex (<40th percentile) and the group with the assumed highest risk due to a relative excess of the binary complex (>60th percentile). The lowest and highest groups were additionally sub-divided with the absolute lowest value <20th percentile (B/T ratio 1.54) and the absolute highest value >80th percentile (B/T ratio 1.62). In the main analysis, the relative risk associated with the B/T ratio >the median was tested with the B/T ratio ≤ median as the reference.

All Cox regression analyses were modelled using a crude model and a model adjusted for confounders: sex (male/female reported in the questionnaire), hypertension (blood pressure >140/90 mmHg and/or treatment for hypertension and/or self-reported), diabetes mellitus (fasting glucose >7.0 and/or treatment for diabetes and/or self-reported), hyperlipidaemia (fasting total serum cholesterol >5.0 and/or treatment for hyperlipidaemia and/or self-reported), smoking, and body mass index (BMI).

In addition, in preliminary analyses estimates were adjusted for treatment with anti-inflammatory agents (high dose aspirin, statins), or immunomodulatory drugs (oral glucocorticoids, Sulfasalazine, Olsalazine, Mesalazine, Azathioprine, Cyclosporine, Methotrexate) as stated in the questionnaire.

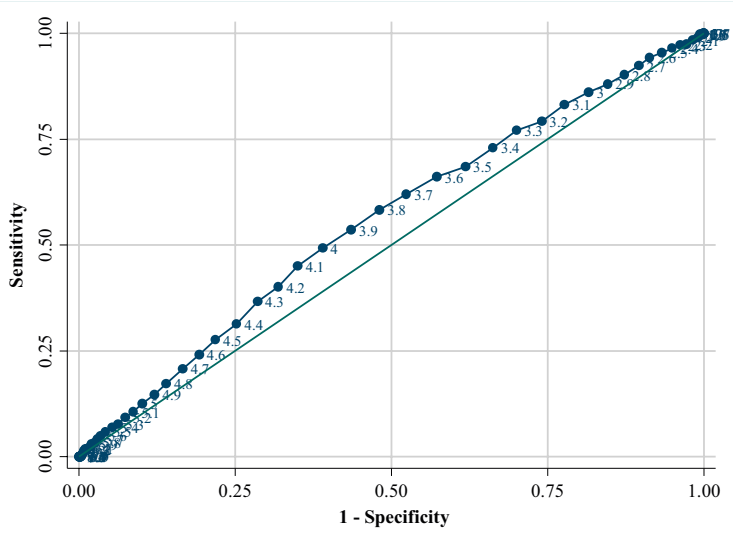
To analyse the potential impact of missing values on the risk estimates, a complete-case analysis was performed. The analysis showed that disparities in crude and adjusted risk estimates did not depend on missing values (data not shown).

We finally tested if the B/T ratio was able to improve discrimination and re-classification measures. The area under the curve (AUC) with a 95% CI and the Net Reclassification Improvement (NRI) were calculated to estimate the incremental discriminatory value of the B/T ratio in predicting future CVE compared with the Framingham Risk Score (FRS) combined with IL6 by means of a published algorithm (207). The FRS estimating 10-year CVE risk was categorised according to the 20% risk while IL6 was categorised in quartiles and the B/T ratio dichotomised at the median.

3.3.2 Study II

In the primary analysis, the risk of future CVE associated with the B/T ratio >the median was analysed stratified by baseline LDL cholesterol levels ≤4.0 and >4.0 mmol/L. The 4.0 mmol/L cut-off was derived from the receiver-operating characteristics (ROC) curve and the sensitivity and specificity for different cut-offs were tested (**Figure 9**).

Figure 9. ROC curve for LDL-cholesterol as predictor of CVE



AUC (95% CI) 0.558 (0.531-0.584)

LDL	Sensitivity (%)	Specificity (%)
2.9	90.22	12.71
3.0	88.06	15.35
3.1	86.11	18.42
3.2	83.17	22.29
3.3	79.26	25.95
3.4	77.10	29.95
3.5	72.99	33.74
3.6	68.49	38.14
3.7	66.14	42.70
3.8	62.04	47.59
3.9	58.32	51.92
4.0	**53.62**	**56.42**
4.1	49.32	60.98
4.2	45.01	64.99
4.3	40.12	68.09
4.4	36.59	71.41
4.5	31.31	74.77

Figure 9. *Receiver Operating Characteristics (ROC) Curve of LDL as predictor of CVE in the 60YO.*

Reproduced with permission from Ziegler et al, The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events, European Journal of Preventive Cardiology. 2020;27:122-129 (Study II).

The CVE risk associated with B/T ratio >median was in additional analyses estimated in sub-groups of low LDL: LDL < 3.0 and ≥3.0–≤4.0 and in subjects at low (< 10%), intermediate (10-20%) and high (>20%) 10-year CVE risk according to the FRS classification (208). The risk factors included in the FRS are: sex, age, total cholesterol/HDL-cholesterol ratio, systolic blood pressure, smoking, diabetes mellitus, and hypertensive treatment.

Cox proportional hazards model was used to estimate CVE risk associated with the B/T ratio > median. Relative risk was expressed as HR with 95% CI and B/T ratio ≤ median was the reference group in all analyses. Schoenfeld's test confirmed proportionality of hazards.

All estimates of relative risk or time to event were analysed as a crude model and adjusted for the same cardiovascular risk factors as in Study I (sex, BMI, current smoking, hypertension and diabetes mellitus). In addition, to test for potential confounding, reported ongoing long-term lipid lowering (n= 126, out of which 72 on statins) or immunomodulatory treatment (n=152) or aspirin in >250 mg doses (n=31) was added to the multivariable model.

Differences in time to event between groups defined by the dichotomised B/T ratio and stratified by LDL levels were expressed in years (95% CI) and estimated using quantile regression for censored data, implemented with Laplace regression (209). This quantile regression method models any given percentile of survival or as in our analysis, failure e.g. the 10th percentile of failure (CVE), as a function of covariates such as the B/T ratio. For example, the 10th percentile of failure is the point in time by which 10% of the cohort has had CVE. Since approximately 10% of the participants in our LDL stratified analysis had experienced CVE during follow-up, we estimated the 5^{th} and 10^{th} percentiles of CVE. We then tested whether these two time points in subjects with a B/T ratio >median differed from that in those with lower B/T ratio levels after adjusting for the above-mentioned confounders.

In secondary analyses, the risk of CAD and ischaemic stroke in addition to time to CAD and ischaemic stroke associated with B/T ratio > median vs. ≤median was estimated in the two LDL groups (≤4.0 and >4.0). Incident CAD cases were excluded when analysing the risk of ischaemic stroke similarly as ischaemic stroke cases were excluded when analysing the risk of CAD events associated with the B/T ratio.

With the close interaction between inflammation and cholesterol in atherosclerosis initiation and progression the effect of potential interaction between the B/T ratio and LDL levels on the risk of CVE was estimated by means of a model of biological interaction on an additive scale (210). We calculated the interaction

between LDL levels >4.0 mmol/L and B/T ratio >median through the estimation of the relative excess risk due to interaction (RERI), the attributable proportion (AP) and the synergy index (SI). The biological interaction assumes that if two factors are interacting, the risk associated with the presence of both is higher than the sum of the risk associated with each one of them. To model this interaction four exposure groups were defined according to LDL levels and the B/T ratio a: the reference group (LDL ≤4.0;B/T ratio ≤median), the group exposed to LDL >4.0 but B/T ratio ≤median; the group exposed to B/T ratio >median but LDL ≤4.0; and finally the group exposed to LDL >4.0;B/T ratio >median. In the interaction model, the relative risk of CVE expressed as HR (95% CI) associated with the singular exposure of B/T ratio (B/T ratio >median;LDL ≤4.0), singular exposure of LDL (LDL >4.0;B/T ratio ≤median) and the combined exposure of B/T ratio and LDL (B/T ratio >median;LDL >4.0), is estimated compared to the reference group (B/T ratio ≤median;LDL ≤4.0). When RERI and AP are equal to 0 and SI simultaneously is equal to 1 there is no interaction (211).

The area under the receiver-operating characteristics curve (AUC) was calculated in subjects with low compared to high LDL (≤4.0 and >4.0) to estimate the incremental discriminatory value of the B/T ratio i.e. if the B/T ratio improved discrimination of cases from non-cases. The B/T ratio was added to validate markers of CVE risk including the FRS and IL6, both included as continuous variables.

3.3.3 Study III

In the primary analysis, differences between symptomatic and asymptomatic patients with regard to IL6, sIL6R and sgp130 plasma concentrations and *IL6*, *IL6R*, *sIL6R*, *GP130*, *sGP130* gene expression in carotid artery plaques were analysed by means of Kruskal-Wallis rank test.

In addition, differences in plasma levels and gene expression depending on to time passed from symptom to surgery (symptomatic patients) was analysed with the time variable dichotomised at 30 days.

In secondary analyses, the correlation between plasma levels of IL6, sIL6R and sgp130 and plaque gene expression levels of *IL6*, *IL6R*, *sIL6R*, *GP130*, *sGP130* was analysed using Spearman's rank correlation coefficient, rho.

Furthermore, differences in plasma and plaque gene expression levels of IL6 and the receptors in statin and non-statin treated patients were analysed. Two individuals were excluded from the latter analysis: monotherapy with Ezetimibe (n=1) i.e. neither statin treatment nor free of lipid lowering agent and missing information on lipid lowering therapy (n=1).

3.3.4 Study IV

Cox proportional hazards model was used to assess the risk of future ischemic stroke associated with IL6 trans-signalling, estimated by the B/T ratio dichotomised at the median, with risk expressed as HR with 95% CI.

The risk of future ischemic stroke in participants with and without prevalent or incident AF was analysed in the primary analysis. Estimates were presented crude and adjusted for the same cardiovascular risk factors as in Study I and II (sex, BMI, hypertension, diabetes mellitus, hypercholesterolemia and smoking) and in addition long-term anticoagulant treatment reported at baseline. Anticoagulants were categorised based on the codes from the Anatomic Therapeutic Chemical classification system, ATC: B01AA (vitamin K antagonists e.g. warfarin) or B01AB (heparin group).

The risk of incident AF associated with the B/T ratio was analysed in secondary analyses. To analyse the nature of the association between the B/T ratio and the outcome, incident AF, the B/T ratio was initially categorised in quartiles and then dichotomised at the median. In addition, the association between IL6, sIL6R and sgp130, components in the B/T ratio, and the risk of AF was investigated.

In secondary analyses, regression analyses were adjusted for sex, hypertension, BMI, and left ventricular hypertrophy (LVH). LVH was defined based on standard 12-lead resting ECG and using the validated criteria of the Minnesota Code and the Cornell voltage–duration product. Either one of the two diagnostic scores had to be positive for the ECG findings to be defined as LVH. To validate the LVH categorisation, 10% of ECG samples were randomly selected (n=400) and evaluated by Professor Sverker Jern's research group in Gothenburg, Sweden. There was 83% correspondence between the two ECG assessments (212).

A sensitivity analysis was performed to analyse the nature of the association when excluding IL6 values >20 pg/mL (n=18) potentially mirroring other types of inflammatory or infectious conditions.

Given the effect of age on the risk of AF, cumulative incidence of AF was presented graphically in Kaplan Meier curves stratified by the B/T ratio dichotomised at the median. In addition, cumulative incidence graphs were presented stratified by quartiles of IL6, sIL6R and sgp130. As IL6 was the only one of the three IL6 trans-signalling components that displayed a trend towards a higher AF incidence with increasing concentrations in particular above the 75th percentile, this marker was stratified by the 75th percentile presented in an additional graph. Given that survival curves cannot take into account potential confounders, quantile regression for censored data implemented using Laplace regression analysis, expressed in years with a 95% CI was applied to adjust for the above-mentioned confounders.

4 RESULTS

4.1 Study I

Table 3 shows the most relevant clinical characteristics of the study participants from the 60YO cohort (n=3624) included in the Study I. Incident cases show a higher proportion of individuals exhibiting the traditional cardiovascular risk factors. In addition, levels of IL6, sIL6R and sgp130 were significantly higher in cases of CVE compared to the referents (p=0.0001 for IL6 and sIL6R and 0.005 for sgp130).

Table 3. Clinical characteristics of the study population at baseline.

	Cases (n=525)	**Referents** (n=3099)
Female (%)	36.2	55.7
Systolic blood pressure, mm Hg	144.5 (132-159.5)	134.5 (121-150)
Diastolic blood pressure, mm Hg	87.5 (81.5-94.5)	82.5 (76-90)
BMI, kg/m^2	27.0 (24.5-29.8)	26.1 (23.7-28.8)
Biochemical measurements (mmol/L)		
LDL cholesterol	4.0 (3.4-4.6)	3.8 (3.2-4.5)
Fasting glucose	5.3 (4.9-5.9)	5.2 (4.8-5.6)
CV risk factors (%)		
Hypertension	20.2	15.0
Hypercholesterolemia	4.2	3.4
Diabetes mellitus	6.3	2.3
Current smoking	29.2	19.8
IL6 system		
IL6 (pg/mL)	0.64 (0.46-0.95)	0.57 (0.41-0.86)
sIL6R (ng/mL)	36.7 (29.6-45.4)	32.8 (26.7-40.7)
sgp130 (ng/mL)	391.5 (334.3-459.4)	381.4 (317.5-448.9)

Continuous data are presented as median (IQR) and categorical data are presented as percentages. Missing values (available in cases/referents): systolic and diastolic blood pressure n=3 (525/3096), LDL n=45 (511/3068) and smoking n=44 (520/3060).

Reproduced with permission from Ziegler et al, Interleukin 6 trans-signalling and risk of future cardiovascular events, Cardiovascular Research. 2019;115:213-221 (Study I).

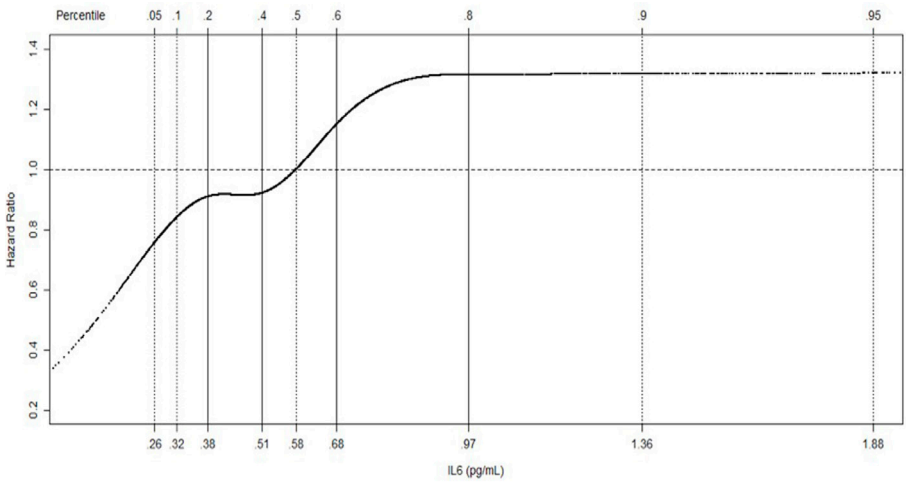
We analysed of the association of each component in IL6 trans-signalling with the risk of CVE: IL6 and sIL6R showed a linear association (**Figure 10, Panel A and B**).

In the adjusted analysis, 0.1 pg/mL IL6 serum level increase was associated with CVE with an HR 1.00 and 95% CI 0.996–1.003, whereas 1 ng/mL sIL6R increase was associated with CVE with an HR 1.02 and 95% CI 1.01–1.02.

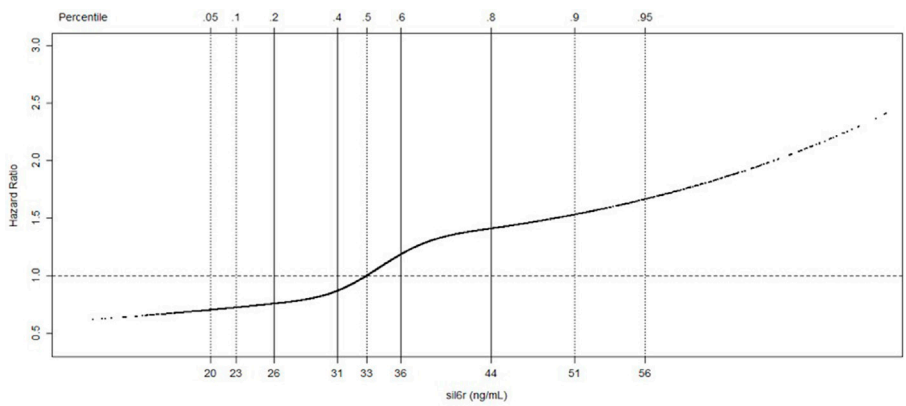
On the other hand, sgp130 exhibited a non-linear association with the CVE risk, as seen in **Figure 10, Panel C**. CVE risk increased progressively with increasing sgp130 concentrations up to the 75th percentile and slowly decreased at higher serum levels. In particular, levels above the 90th, and even more so above the 95th, percentile were associated with a CVE risk reduction.

Figure 10. Graphical representation of the association of serum IL6 concentrations (Panel A), sIL6R (Panel B) and sgp130 (Panel C) with time to first-time CVE modelled using natural cubic splines.

Panel A.



Panel B.



Panel C.

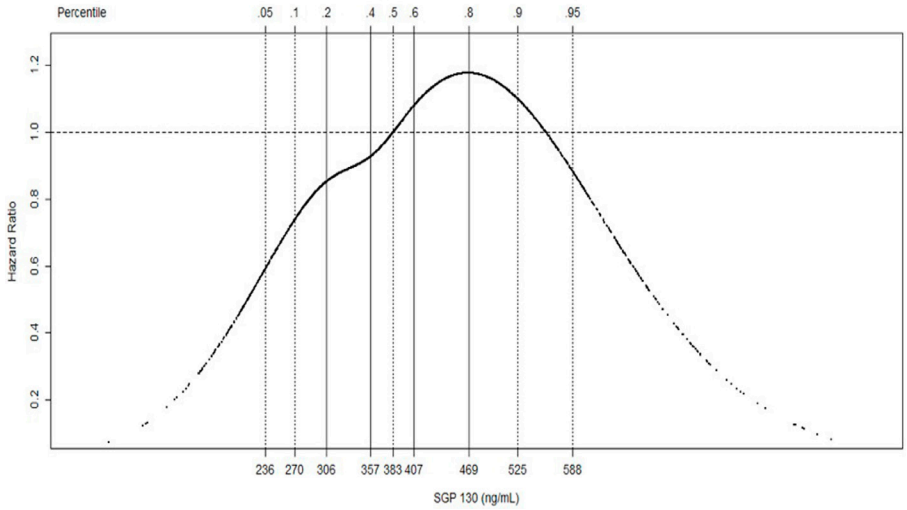


Figure 10. *Graphical presentation of the association between increasing serum concentrations of IL6 (pg/mL) (Panel A), sIL6R (ng/mL) (Panel B), and sgp130 (ng/mL) (Panel C) with the risk of CVE estimated by Cox regression using natural cubic splines and expressed as hazard ratio.*

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While these analyses were relevant to understand the relationship of each biomarker with the CVE risk, they did not give us any information on the association of the IL6 trans-signalling and CVE risk. To analyse whether IL6 trans-signalling associates with the CVE risk, we estimated the circulating levels of the binary (IL6:sIL6R) and ternary (IL6:sIL6R:sgp130) complexes taking into account the molar concentration. The three biomarkers interact in fact on a molar basis and this transformation is necessary to estimate their relative amounts. From the binary and ternary complex, we calculated the ratio between the two, the B/T ratio, under the assumption that an excess of the binary complex or of the ternary complex could be associated with the outcome.

Our analysis shows that B/T ratio had a monotone association with CVE risk (**Figure 11**). For every increase of 0.1 unit of the B/T ratio the CVE risk increased with a crude HR 1.45 and 95% CI 1.28–1.64 and an adjusted HR 1.31 and 95% CI 1.13–1.51 ($p < 0.001$ in both models).

The results from the natural cubic splines analysis indicated that the median was an adequate cut-off with a risk decrease observed with B/T ratio levels <median (relative excess level of the ternary complex) and increased risk with levels >median

(relative excess level of the binary complex). With the median as cut-off, there was an increased CVE risk associated with the B/T ratio >median compared to the reference (≤median) with an adjusted HR 1.44 and 95% CI 1.21–1.72 (**Table 4, lower section**).

Figure 11. The binary/ternary complex ratio association with CVE

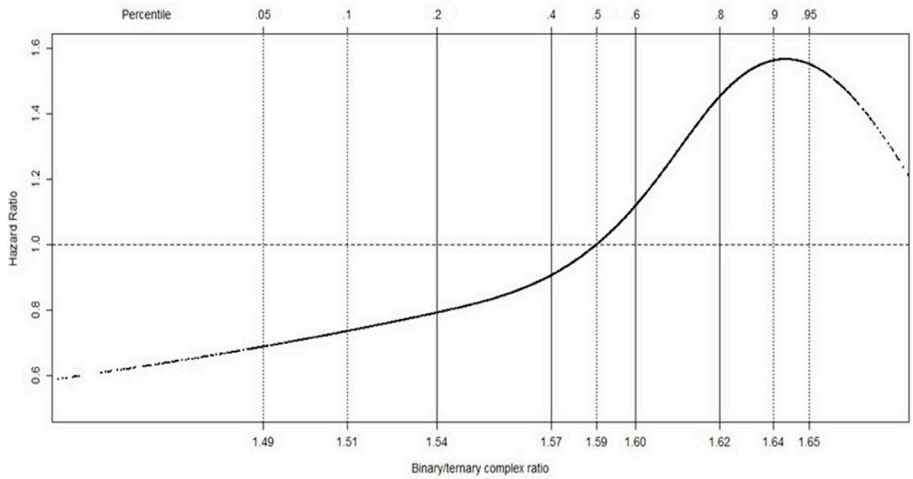

Figure 11. *Graphical presentation of the association between increasing serum values of the binary/ternary complex ratio with the risk of CVE estimated by Cox regression using natural cubic splines and expressed as hazard ratio.*

Table 4. Risk of CVE associated with the B/T ratio

Binary/ternary complex ratio	**N case/ref** All: 525/3099	**Crude** (n=3624)	**P**	**Adjusted** (n=3580)	**P**
< 20th	73/652	0.74 (0.55-1.01)	0.06	0.82 (0.60-1.11)	0.19
20-40th	86/639	0.88 (0.66-1.18)	0.41	0.89 (0.66-1.19)	0.43
40-60th	96/629	1 (ref)		1 (ref)	
60-80th	130/595	1.39 (1.07-1.81)	0.02	1.30 (1.00-1.70)	0.05
80-100th	140/584	1.51 (1.17-1.96)	0.002	1.29 (0.99-1.67)	0.06
≤median	205/1607	1 (ref)		1 (ref)	
>median	320/1492	1.63 (1.37–1.95)	<0.001	1.44 (1.21–1.72)	<0.001

Adjusted model adjusted for sex, BMI, hypertension, diabetes, hypercholesterolemia and smoking. The adjusted model contains fewer subjects (cases=520) due to missing values as described in Table 1. Reference group for the binary/ternary complex ratio: 40-60th percentiles in first analysis and ≤median in the second. Ref=references i.e. subjects without a CVE.

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We analysed this association further, looking into categories of the B/T ratio (<20th; 20-40th; 40–60th; 60-80th and >80th percentiles) with the category containing the median (40-60th percentiles) as the reference group. There was an indication of a decreased CVE risk associated with the B/T ratio <40th percentile (representing a relative excess of the ternary complex) although this association was not significant. B/T ratio levels >60th percentile (representing an excess of the binary complex) were on the other hand associated with a 30% risk increase with a close to doubled number of cases compared to the two low B/T ratio groups (**Table 4, top section**).

In addition, adjusting for medical treatment, drugs affecting inflammation did not change the risk estimates (data not shown).

The same pattern of association between the B/T ratio CVE risk was seen assuming ten times higher and lower dissociation constants (**Figure 12**).

Figure 12. Graphical presentation of risk associated with the binary/ternary complex ratio with different dissociation constants.

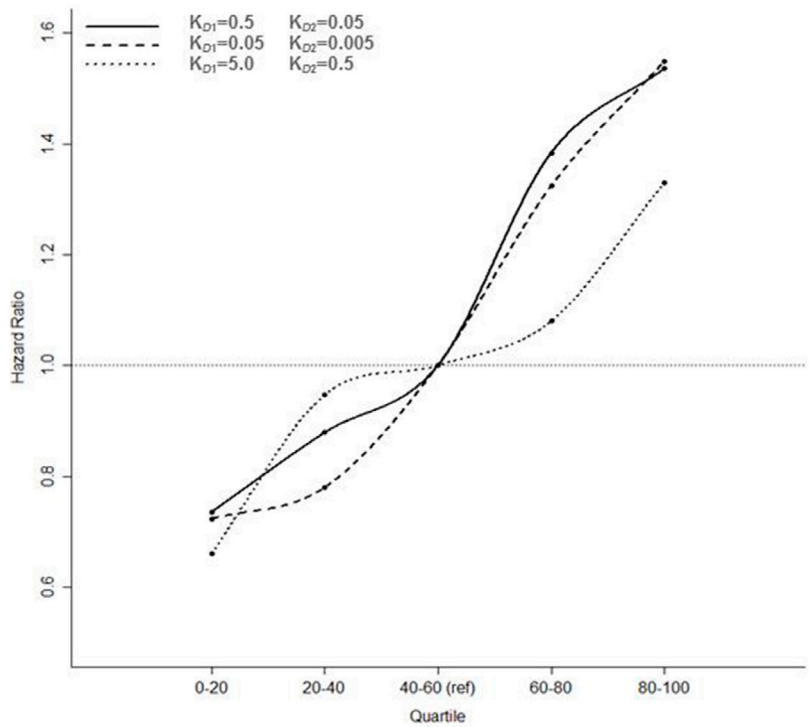

Figure 12. *Risk of CVE associated with increasing levels of the binary/ternary complex ratio estimated by Cox regression assuming different dissociation constants for the binary (K_{D1}) and ternary complex (K_{D2}). The dissociation constants are expressed in nM.*

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A modest increment in the discriminatory value measured with the AUC was the result of adding the B/T ratio to the FRS and IL6: FRS + IL6 + B/T ratio 0.62 (0.59–0.64) vs. FRS + IL6 0.59 (0.56–0.62). In the reclassification analysis, adding the B/T ratio to FRS + IL6 reclassified 10% of the patients correctly (NRI= + 0.10, P=0.006 for FRS + IL6 + B/T ratio vs. FRS + IL6).

4.2 Study II

The study population's clinical characteristics are described in **Table 3 (Study I)**.

The analyses in this study follow our observations in Study I. Given the relevance of LDL-cholesterol in determining CVE risk, we sought to investigate if a B/T ratio >median could be a predictor of risk in the presence of LDL ≤4.0, the most suitable cut-off to predict LDL associated increased CVE risk in our population based the ROC curve and sensitivity and specificity analyses. In primary analyses we estimated the risk of CVE associated with a B/T ratio >median in study participants with LDL ≤4.0 compared with the CVE risk in those with higher LDL. We observed that the highest CVE risk was observed in participants with low LDL (HR 1.59 vs 1.29) in the presence of B/T> median, as seen in **Table 5**.

Table 5. Risk of CVE associated with the B/T ratio > median and stratified by LDL levels.

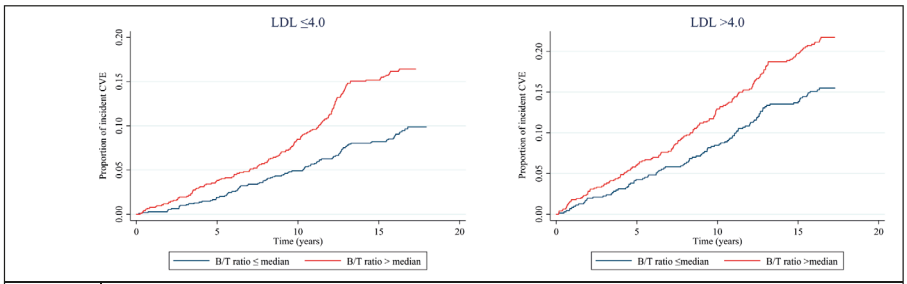
Type of event	Crude			Adjusted		
	HR (95% CI)	p	N (case /ref)	HR (95% CI)	p	N (case/ ref)
Composite CVE (n=525)						
LDL ≤4.0	1.79 (1.39-2.29)	<0.001	259/1871	1.59 (1.24-2.05)	<0.001	257/1850
LDL >4.0	1.45 (1.13-1.87)	0.004	252/1197	1.29 (1.00-1.67)	0.049	249/1179

The risk of composite CVE associated with the B/T ratio > vs. ≤median estimated by Cox regression expressed as HR (95% CI). In the adjusted analysis estimates were adjusted for sex, BMI, hypertension, diabetes mellitus and smoking. Reproduced with permission from Ziegler et al, The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events, European Journal of Preventive Cardiology. 2020;27:122-129 (Study II).

In addition, the difference in time to CVE associated with a B/T ratio >median vs. ≤median was analysed according to LDL levels. B/T ratio >median was associated with earlier onset of CVE in individuals with LDL ≤4.0 (**Figure 13**, left panel) as compared to those with B/T ratio≤median. At the time-point when 10% of the

study population with LDL ≤4.0 had experienced a CVE, after around16 years of follow-up, subjects with a B/T ratio >median had their CVE four years earlier. In the group with LDL>4.0, presence of B/T ratio> median did not associate with earlier debut of CVE (**Figure 13**, right panel).

Figure 13. Differences in time to CVE with the B/T ratio stratified by LDL



Proportion CVE	Time in years (95% CI) to CVE with B/T ratio > median and low/high LDL levels							
	LDL ≤ 4.0				LDL > 4.0			
	Crude	P	Adjusted	P	Crude	P	Adjusted	p
5%	-3.3 (-0.5; -6.0)	0.02	-1.4 (1.6; -4.4)	0.35	-1.7 (0.3; -3.7)	0.09	-0.3 (2.4; -3.1)	0.82
10%	-5.5 (-3.6; -7.5)	<0.001	-3.9 (-2.0; -5.8)	<0.001	-2.7 (-0.6; -4.8)	0.01	-2.5 (0.3; -5.3)	0.08
15%	NA	-	NA	-	-4.1 (-1.2; -6.9)	0.005	-1.5 (0.6; -3.6)	0.17

Figure 13. *Difference in years (95% CI) to CVE with B/T ratio > median vs. ≤ median in subjects with LDL ≤4.0 and >4.0 mmol/L. Case/referent numbers in the crude analysis: LDL ≤4.0 group: 259/1871 (14% cases), and LDL >4.0 group: 252/1197 (21% cases). Missing data on LDL n= 45. NA= not applicable. Reproduced with permission from Ziegler et al, The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events, European Journal of Preventive Cardiology. 2020;27:122-129 (Study II).*

To further analyse the association between the B/T ratio and CVE in individuals with low-intermediate cardiovascular risk we went on to investigate the effect B/T ratio stratified by LDL with an additional cut-off at a lower level creating three groups; LDL<3.0 (n=532), LDL 3.0–4.0 (n=1598) and LDL >4.0 (n=1494). As the optimal discriminatory value in our cohort was LDL 4.0, the lower cut-off has a lower discriminatory value in addition to the sub-group being small with few cases (n=61). We did however see an increased relative risk of CVE associated with a B/T ratio >median in the two sub-groups below 4.0 mmol/L, albeit not statistically significant in the small <3.0 mmol/L group (**Table 6**).

Table 6. CVE risk associated with B/T ratio> median stratified by LDL <3.0, ≥3.0-≤4.0, >4.0 mmol/L.

LDL (mmol/L)	N ref/case	Crude HR (95% CI)	P	Adjusted HR (95% CI)	P
< 3.0	471/61	1.74 (1.05-2.91)	0.03	1.45 (0.86-2.47)	0.16
≥3.0-≤4.0	1400/198	1.80 (1.35-2.40)	<0.001	1.64 (1.22-2.19)	0.001
> 4.0	1197/252	1.45 (1.13-1.87)	0.004	1.29 (1.00-1.67)	0.049

Ref/case: referents/incident cases. Risk of CVE expressed as hazard ratio (HR) with 95% confidence interval (CI). Multivariate analysis adjusted for sex, BMI, hypertension, diabetes and smoking. Reproduced with permission from Ziegler et al, The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events, European Journal of Preventive Cardiology. 2020;27:122-129 (Study II).

In addition, we analysed the risk of CVE associated with a high B/T ratio in subjects with low, intermediate and high 10-year risk of CVE according to the FRS and found that the B/T ratio was associated with an increased CVE risk only in participants classified as at low or intermediate risk by the FRS (**Table 7**).

Table 7. CVE risk associated with B/T ratio in individuals with low, intermediate and high CVE risk defined by the Framingham risk score.

10-year CVE risk	N ref/case	HR (95% CI)	P
≤ 10%	2287/274	1.27 (1.00-1.61)	0.047
> 10%-≤ 20%	632/158	1.78 (1.36-2.34)	<0.001
> 20%	138/88	1.48 (0.95-2.32)	0.087

Ref/case: referents/incident cases. Risk of CVE expressed as HR (95% CI). Missing values for the FRS=47. Given that the cardiovascular confounders incorporated in the multivariate analyses are all included in the FRS, estimates presented in the table are crude. Reproduced with permission from Ziegler et al, The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events, European Journal of Preventive Cardiology. 2020;27:122-129 (Study II).

In secondary analyses, we analysed the B/T ratio associated with relative risk of coronary events (n=361) and ischaemic stroke (n=164) separately and in addition the difference in time to the respective event in relation to the B/T ratio. All analyses were stratified by LDL. The B/T ratio was solely associated with an increased risk of having future CAD (HR 1.52; 95% CI 1.11–2.08) and ischaemic stroke (HR 1.84; 95% CI 1.19–2.83) amongst subjects with LDL ≤4.0 while no association was seen for either type of event in those with higher LDL (**Table 8**).

Table 8. Risk of coronary events and ischemic stroke associated with the B/T ratio >median stratified by LDL levels.

Type of event	Crude			Adjusted		
	HR (95% CI)	p	N (case /ref)	HR (95% CI)	p	N (case/ ref)
Coronary events (n=361)						
LDL ≤4.0	1.79 (1.32-2.44)	<0.001	170/1871	1.52 (1.11-2.08)	0.009	168/1850
LDL >4.0	1.44 (1.07-1.94)	0.015	184/1197	1.28 (0.95-1.73)	0.10	182/1179
Ischemic Stroke (n=164)						
LDL ≤4.0	1.86 (1.21-2.85)	0.004	89/1871	1.84 (1.19-2.83)	0.006	89/1850
LDL >4.0	1.58 (0.97-2.57)	0.07	68/1197	1.41 (0.86-2.31)	0.18	67/1179

The risk of coronary events and ischemic stroke associated with the B/T ratio > vs. ≤median estimated by Cox regression expressed as HR (95% CI). In the adjusted analysis estimates were adjusted for sex, BMI, hypertension, diabetes mellitus and smoking.

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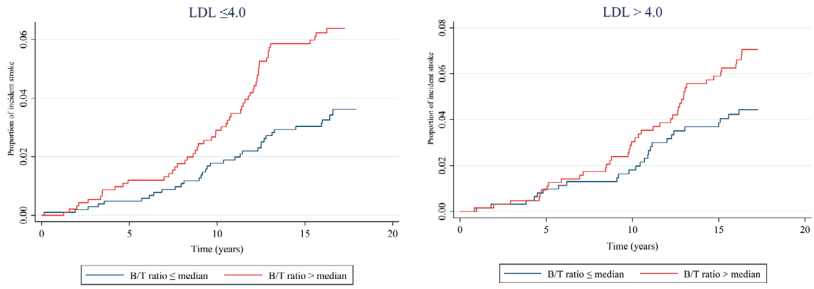
In the time to event analysis, CAD events occurred significantly earlier (3–4 years) with a B/T ratio >median irrespective of the LDL level albeit the association was stronger in the group with lower LDL ($p<0.001$ compared to $p=0.03$). Individuals with ischaemic stroke on the other hand experienced earlier events solely in the LDL ≤4.0 group (3.8 years, $p=0.02$) as seen in **Figure 14**.

We then tested the effect of the interaction between LDL-cholesterol levels and B/T ratio on the risk of CVE. These two factors are biologically related in the progression of atherosclerosis, however our data seemed to indicate a specific pattern of association were the B/T ratio was able to predict CVE risk in subjects with low-normal cholesterol levels.

Figure 14. Differences in time to ischemic stroke associated with the B/T ratio stratified by LDL-cholesterol levels ≤ 4.0 and >4.0 mmol/L.



Proportion stroke	Time to ischemic stroke with B/T ratio > median and LDL ≤ or > 4.0 mmol/L							
	LDL ≤ 4.0				LDL > 4.0			
	Crude	p	Adjusted	p	Crude	P	Adjusted	p
2.5%	-3.3 (-0.7; -6.0)	0.01	-3.7 (2.1; -9.5)	0.21	-1.2 (0.7; -3.0)	0.21	-0.2 (43.1; -43.5)	0.99
5%	-6.5 (-4.1; -8.9)	< 0.001	-3.8 (-0.7; -6.8)	0.02	-4.3 (-1.5; -7.2)	0.003	-1.5 (2.1; -5.0)	0.42

Figure 14. *Differences in time (years; 95% CI) to ischemic stroke with the B/T ratio in subjects with LDL ≤4.0 and >4.0 mmol/L. Case/referent numbers in the adjusted analysis: 89/2018 in LDL ≤ 4.0 and 67/1361 in LDL >4.0. Reproduced with permission from Ziegler et al, The predictive role of interleukin 6 trans-signalling in middle-aged men and women at low-intermediate risk of cardiovascular events, European Journal of Preventive Cardiology. 2020;27:122-129 (Study II).*

The risk of CVE associated with exposure to IL6 trans-signalling alone or LDL alone or both in combination was compared to the reference group with no exposure of either one. The regression analysis displayed a comparable risk increase when either of the two exposures; B/T ratio and LDL, was present (HR 1.58; 95% CI 1.23 -2.04 and HR 1.68; 95% CI 1.27 -2.21, respectively). The combination of the two exerted the highest CVE risk (HR 2.17; 95% CI 1.68–2.80) as shown in **Figure 15.** The risk associated with both LDL and the B/T ratio was however not higher than the sum of the risk associated with LDL or the B/T ratio alone. Together with RERI -0.04, AP -0.01 and SI 0.97 with p=0.9, this suggests that the two exposures do not interact.

To test the incremental discriminatory value of the B/T ratio, the AUC for the continuous FRS and IL6 plus the addition of the B/T ratio was analysed. In the unstratified cohort the AUC including the B/T ratio was 0.67 while when stratifying by low or high LDL (≤ or >4.0 mmol/L), the AUC was 0.68 in the LDL ≤4.0 group and 0.65 in the >4.0 group, indicating an increased incremental value adding the B/T ratio to existing risk markers in groups of low-normal LDL values.

Figure 15. The risk of CVE associated with the B/T ratio and LDL

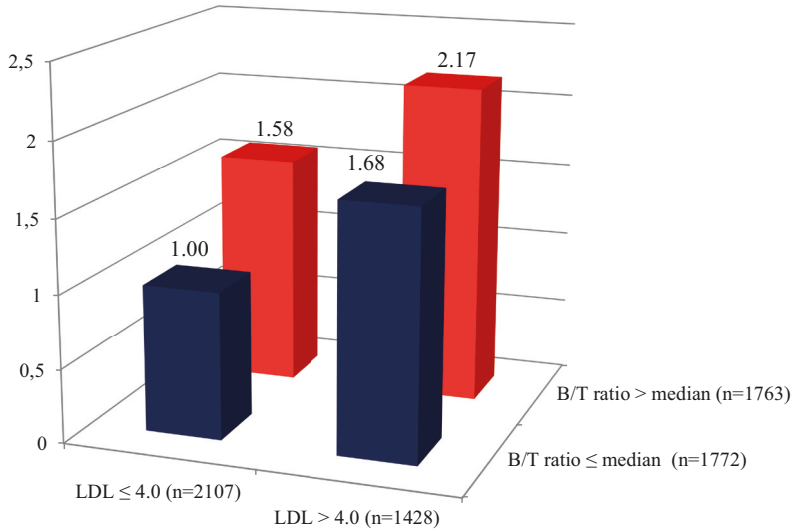


Figure 15. *The risk of CVE associated with the B/T ratio alone (B/T ratio >median; LDL ≤4.0 mmol/L), LDL alone (LDL >4.0 mmol/L; B/T ratio ≤median), B/T ratio in combination with LDL (B/T ratio >median; LDL >4.0 mmol/L) compared to the reference group (B/T ratio ≤median; LDL ≤4.0 mmol/L). Risk calculated by Cox proportional hazards model presented as hazard ratio. All p <0.001. Missing data: LDL n=45 and smoking n=44.*

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4.3 Study III

The clinical characteristics of the BiKE study population analysed in the present study is summarised in **Table 9**. Among asymptomatic patients there were more men, hypertensives and subjects with overweight while symptomatic participants were older.

Table 9. Clinical characteristics of the study population stratified stroke symptoms preceding CEA

	All (n=78)	Symptomatic (n=53)	Asymptomatic (n=25)
Age (years)	73 (65-78)	76 (68-80)	68 (60-73)
Males (%)	74.4	64.1	96.0
Hypertension (%)	85.9	81.1	96.0
Diabetes mellitus (%)	29.5	28.3	32.0
Statin treated (%)	86.3	80.0	89.1
Overweight (%)	54.2	46.8	68.0
Smoking (%)	26.5	26.1	27.3

Missing information on statin treatment (n=2).

The analysis of the expression of *IL6, IL6R, sIL6R, GP130* and *sGP130* shows that all the genes were expressed in carotid plaques. Moreover, both membrane-bound receptor genes (*GP130* and *IL6R*) displayed a higher expression level than their respective soluble form (*sGP130* and *sIL6R)* as seen in **Figure 16**.

Figure 16. IL6 signalling gene expression levels in carotid artery plaques

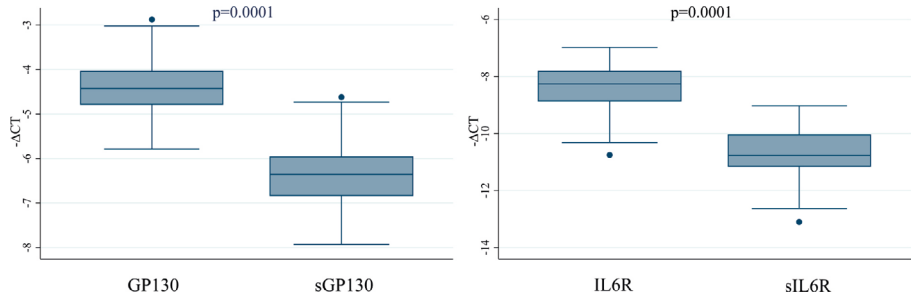

Figure 16. *Relative gene expression levels of GP130, sGP130, IL6R and sIL6R in carotid plaques (n=78) measured by semi-qRT-PCR and reported as - ΔC_T. P-value for relative differences in expression.*

Comparing gene expression levels in symptomatic and asymptomatic patients, *IL6R* displayed a higher expression level in symptomatic patients as compared to asymptomatic (p=0.007). The same trend was seen for *sIL6R*, notwithstanding without statistical significance (p=0.06). Contrarily, *GP130* and *sGP130* exhibited higher expression levels in asymptomatic patients as compared to symptomatic without attaining statistical significance (p=0.05 and p=0.08, respectively) (**Figure 17**). On the other hand, *IL6* expression levels did not differ between symptomatic and asymptomatic patients (*IL6* log2 median: 6.46 vs 6.18, p=0.37).

To assess whether potential differences could be attributed to the time span from symptom to surgery, time from symptom to CEA was incorporated as a factor in the analysis. Gene expression and plasma levels were stratified by time from symptom to surgery in symptomatic patients resulting only in higher sIL6R plasma levels in those with symptoms ≤30 days before CEA as compared to those with symptoms >30 days (median 73.3 vs 55.8 ng/mL, p=0.02) before CEA. No difference was observed for IL6, sIL6R, sgp130 or hsCRP plasma levels.

In secondary analyses, we explored the correlation between plasma levels of each biomarker and plaque gene expression levels. Our results show a moderate and borderline significant positive correlation between plasma IL6 and sIL6R with of *IL6* and *sIL6R* (both p=0.05), respectively. Furthermore, an indication, albeit statistically insignificant, of positive correlations was observed for sIL6R with *GP130* and *sGP130* (**Figure 18**). No differences in correlations could be detected comparing symptomatic and asymptomatic subjects (data not shown).

Figure 17. IL6 signalling gene expression levels in symptomatic and asymptomatic plaques

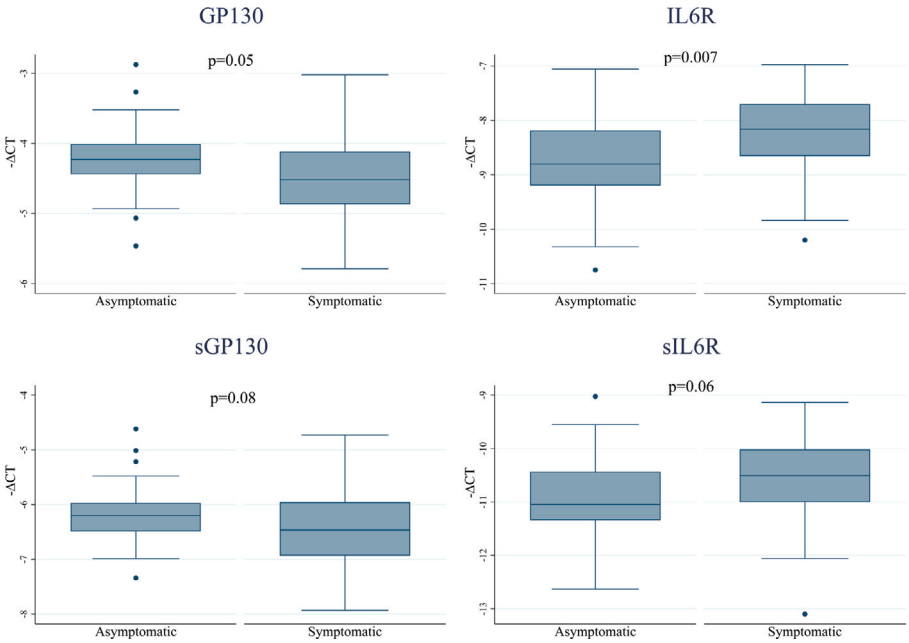

Figure 17. *Relative gene expression levels of GP130, sGP130, IL6R and sIL6R in carotid plaques in asymptomatic and symptomatic study participants, quantified by semi-qRT-PCR and expressed as - ΔC_T. P-value for differences between asymptomatic and symptomatic patients.*

Figure 18. Correlations between plasma levels and plaque expression

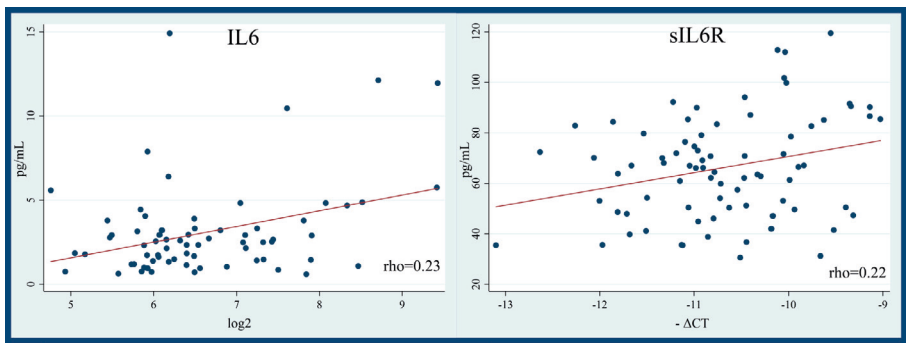

Figure 18. *Scatter plots representing correlations between plasma IL6 and sIL6R levels and gene expression of IL6 and sIL6R in carotid plaques. Correlations were estimated using Spearman's rank correlation coefficient, rho.*

Plasma levels of IL6, sIL6R, sgp130 and hsCRP did not correlate with each other except for IL6 and hsCRP (rho 0.45, p=0.0004).

Given the interplay between cholesterol and inflammation in atherosclerosis, additional analyses were performed to address differences in statin-treated vs. non-treated patients. In the subset of the cohort analysed in this thesis, ≥80% of patients were treated with statins. The largest difference between statin-treated and non-treated patients was seen for sIL6R with higher plasma levels in statin-treated patients (sIL6R median 66.96 vs. 60.9 ng/mL, p=0.05). Regarding gene expression, *IL6* expression did not differ while *IL6R, sIL6R* and *sGP130* were relatively more extensively expressed in plaques from patients on statin treatment compared to non-treated (**Figure 19**).

Figure 19. IL6 signalling gene expression levels in statin treated and non-treated

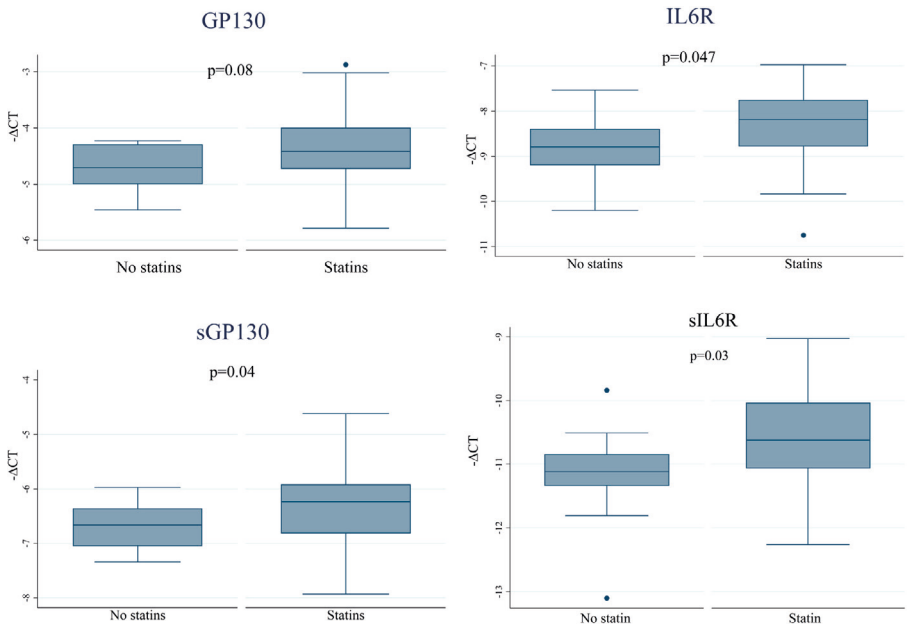

Figure 19. *Relative gene expression of GP130, sGP130, IL6R and sIL6R in carotid plaques stratified by statin treatment (statin treated n=65, no statin n=11) quantified by semi-qRT-PCR and expressed as* $-\Delta C_T$**.** *P-value for in expression between the groups.*

4.4 Study IV

The study population is the 60YO. Clinical characteristics differ slightly from those reported in Study I since the follow-up until December 31st 2017 were available when these analyses were performed. In addition, in this study we focused on ischemic stroke following our observations in Study II, where we observed a stronger association of the B/T ratio with the risk of ischemic stroke and Study III where we had observed that all the component of the IL6 trans-signalling were expressed in the carotid plaques. Therefore, study participants with prevalent and incident CAD have been excluded from the analysis. The clinical characteristics of the study population included in this study are presented in **Table 10**.

After 20-year follow-up, 203 fatal and non-fatal ischemic strokes had occurred. At baseline 29 subjects had prevalent AF whereas 279 individuals got a first-ever diagnosis of AF during follow-up. Participants with prevalent or incident AF suffered more often ischemic strokes than participants without AF. B/T ratio did not differ between the two groups.

Table 10. Baseline characteristics of the study population stratified by case and non-case status

Baseline characteristics	Ischemic stroke (n=203)	No ischemic stroke (n=2990)
Male (%)	116 (57.1)	1309 (43.8)
Hypertension (%)	45 (22.2)	440 (14.7)
Hyperlipidaemia (%)	6 (3.0)	103 (3.4)
Diabetes mellitus (%)	10 (4.9)	76 (2.5)
Atrial fibrillation (%)	5 (2.5)	23 (0.8)
Smoking (%)	56 (27.9)	589 (20.0)
Anticoagulant treatment (%)	1 (0.5)	11 (0.4)
BMI	26.5 (24.4-28.6)	26.0 (23.7-28.8)
Systolic blood pressure	143.5 (130.5-156.5)	134.5 (120.5-150)
Diastolic blood pressure	88 (81-94.5)	82.5 (76-90)
Glucose	5.2 (4.8-5.7)	5.2 (4.8-5.6)
LDL	4.1 (3.4-4.7)	3.8 (3.2-4.5)
b/t ratio	1.60 (1.56-1.63)	1.58 (1.55-1.61)

Continuous variables presented as median (IQR). BMI was expressed as kg/m^2, blood pressure as mm Hg, glucose and LDL as mmol/L. Missing data on: smoking (n=40), LDL (n=37) and blood pressure (n=3).

First we analysed the association of the B/T ratio >median (1.58) with the risk of future ischemic stroke in study participants with and without prevalent or incident AF and observed that in the presence of AF a significant association could not be

demonstrated (adjusted HR 1.54; 95% CI 0.81-2.91), while in subjects without an AF diagnosis a significant association was observed between B/T ratio and risk of ischemic stroke (adjusted HR 1.49; 95% CI 1.08-2.06).

We then analysed the association of the B/T ratio, categorised in quartiles or dichotomised at the median, with the risk of incident AF. The B/T ratio was not associated with the risk of AF in any of these analyses and consistently, no difference in AF prevalence proportion could be seen stratifying by the B/T ratio ≤ and > median throughout the follow up time (**Figure 20, left panel**).

Figure 20. Differences in time to AF diagnosis associated with the B/T ratio

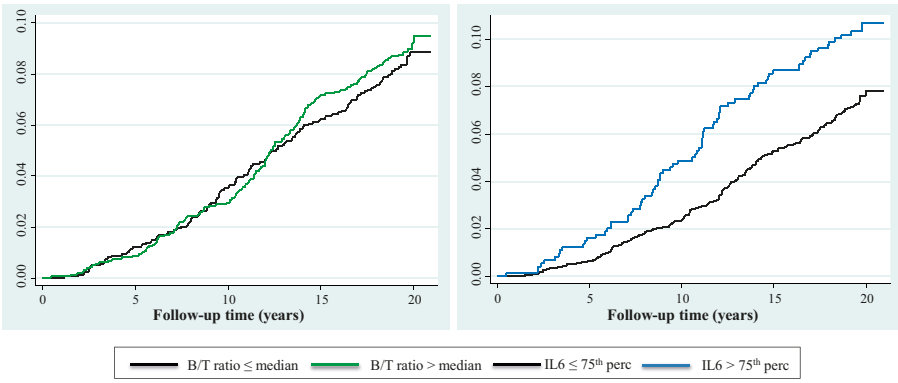

Figure 20. *Kaplan Meier curve of the cumulative atrial fibrillation (AF) incidence in study participants without prevalent AF at baseline and stratified by the B/T ratio (left panel) and IL6 dichotomised at the 75th percentile (right panel).*

As for CVE, we looked into the association of the single components of the IL6 trans-signalling with the risk of AF. Exploring the association between IL6 and AF, the risk estimates suggested increased risk associated IL6 levels >75th percentile albeit without statistical significance (HR1.23; CI 0.93-1.63). When excluding IL6 >20 pg/mL in a sensitivity analysis, a modest risk estimate change was observed (HR1.27; CI 0.96-1.67).

However, we observed that individuals with IL6 levels >75th percentile were diagnosed with AF earlier (**Figure 20, right panel**) than those with lower IL6 levels. At the end of follow-up, the proportions of participants with AF were 6 and 10% for the IL6 ≤ and > 75th percentile, respectively. Applying Laplace regression to quantify the difference in time between the two groups and adjust for confounders, resulted in a 3-year difference (95% CI 0.3-5.7 years, p=0.03) with earlier AF diagnosis in those with the highest IL6 levels at the end of follow-up. The soluble IL6 trans-signalling receptors, sIL6R or sgp130 were not associated with the risk of AF or with an earlier AF diagnosis.

5 DISCUSSION

The main finding of the studies within this thesis is that the IL6 trans-signalling, estimated by the binary/ternary complex ratio (B/T ratio), is associated with atherosclerosis driven coronary and cerebrovascular events.

In Studies I and II, we demonstrate an association between the B/T ratio and an increased risk of future CVE in middle-aged individuals without prevalent CVD, in particular in those with low-intermediate cardiovascular risk based on LDL levels or the FRS. Results from Study II also indicate a stronger association of the B/T ratio with the risk of ischemic stroke.

In Study III, we sought to investigate whether IL6 trans-signalling is present in manifest atherosclerosis. Although far from inferring causality of the association between IL6 trans-signalling and atherosclerosis related CVE, our results provide evidence for the existence of a local IL6 signalling. All IL6 signalling components are expressed in the carotid plaques and in addition they seem to be differentially expressed depending of the instability of the plaque. This may imply the presence of an autocrine/paracrine IL6 trans-signalling that could interact and cooperate with the systemic IL6 trans-signalling in the progression of atherosclerotic plaques.

In Study IV, we have tried to disentangle the role of IL6 trans-signalling as predictor of two common forms of ischemic stroke, the atherothrombotic and cardioembolic. We found that the B/T ratio was associated with the risk of future ischemic stroke only in individuals without AF while no association could be established for ischemic stroke in subjects with AF. Moreover, the B/T ratio was not associated with AF incidence per se. Altogether, our results indicate that IL6 trans-signalling is a relevant and specific predictor of the risk for atherosclerosis related CVE. In addition, we propose a novel biomarker that integrates the components of the IL6 trans-signalling in one entity, the B/T ratio, with high levels representing a relative excess of the active binary IL6:sIL6R complex in relation to the inactivated ternary IL6:sIL6R:sgp130.

Clinical science and practice have come a long way on the cholesterol path in atherosclerosis preventive treatment but has only just entered that of inflammation. The results of the CANTOS trial indicate a possible beneficial effect of IL1β-IL6-CRP pathway inhibition in patients with stable CAD on optimal secondary preventive treatment (12). In sub-analyses of the CANTOS study, it was demonstrated that participants with the highest pre-treatment IL6 concentrations and in addition significant on-treatment decrease in IL6 or hsCRP levels had the greatest effect indicating a central role for IL6 (213, 214).

Similar results were observed in the COLCOT trial where treatment with the anti-inflammatory IL1β inhibiting Colchicine exerts a protective effect on recurrent CVE after recent MI, comparable to that observed with canakinumab (215). When stratifying by event type, the risk reduction was primarily driven by decreased stroke risk.

In spite of the exciting findings from ILlβ inhibition studies, there are negative aspects of inhibiting the entire IL1β-IL6-CRP pathway. The Federal Drug Administration (FDA) has not approved the use of canakinumab in clinical practice because of a high cost/benefit ratio as well as for the increased risk of severe and fatal infections due to the impairment of the immune response (12, 215).

Our studies cannot answer the question on the suitability of IL6 trans-signalling as target for treatment of atherosclerosis related CVE, intervention studies are needed to firmly answer this question. However, given the strong biological evidence linking the IL6 trans-signalling to the inflammatory response in atherosclerosis and the observed associations with the risk of CAD and ischemic stroke reported in our studies, we believe that moving downward in the IL1β-IL6-CRP pathway and targeting the pro-inflammatory IL6 trans-signalling is attractive and worth exploring (**Figure 21**). Targeting IL6 trans-signalling could represent an innovative treatment moiety to modulate inflammation in atherosclerosis avoiding the inhibition of the pivotal IL6 classical signalling and the associated negative effect on the immune response.

Figure 21. Schematic presentation of levels of inhibition of the IL1β-IL6-CRP pathway

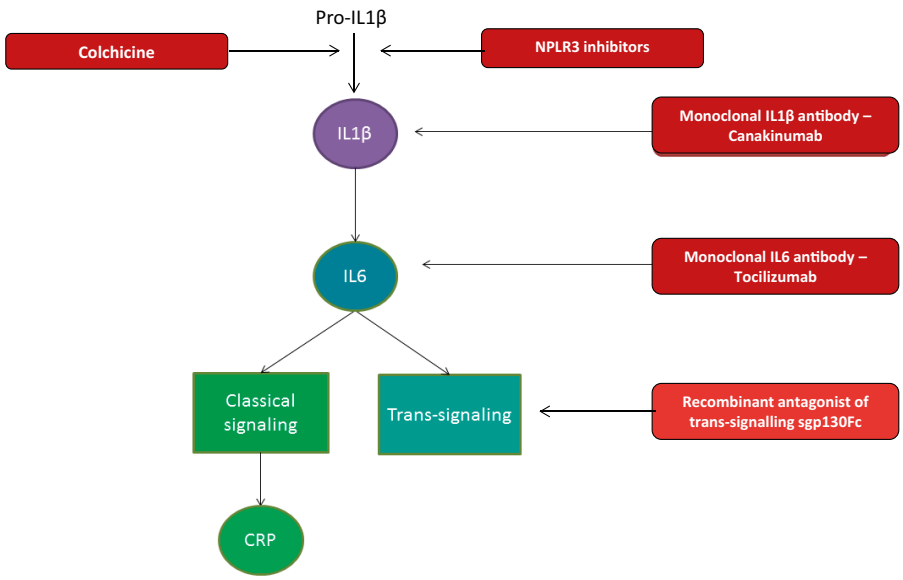

Figure 21. *Adapted with permission from Ziegler et al, Interleukin 6 trans-signalling and risk of future cardiovascular events, Cardiovascular Research. 2019;115:213-221 (Study I).*

5.1 Study I

The main novelty of Study I is the use of the B/T ratio as measure of active IL6 trans-signalling and as a risk predictor for future CVE.

As compared to previous studies, we have for the first time incorporated the active and inactive moieties of this signalling system in one biomarker.

There are some important limitations to this approach. The B/T ratio was calculated from the estimated molar concentration of the binary IL6:sIL6R complex and the ternary IL6:sIL6R:sgp130 complex. The calculation derives from the formulas originally published in a paper by Müller-Newen et al. based on results from *in vitro* studies (75, 101). The rationale underlying this approach is that the interaction among IL6, sIL6R and sgp130 occurs on a molar basis and not on the basis of their mass. However, we cannot exclude that in physiological conditions, the dissociation constants are different from the ones we have used. To verify that the association of the B/T ratio with the outcome holds even with higher or lower dissociation constants, we performed the association analysis assuming that the dissociation constants were 10 times higher and 10 times lower and found that the association withheld. Since this is a prospective analysis, a potential error would have resulted in a non-differential misclassification thus not affecting the risk estimates. In addition, the B/T ratio cut-off was data driven which potentially can prevent generalisability of the results to other populations. Our ambition is however not to disclose reference values and cut-offs for future analyses but rather to explore the association between active IL6 trans-signalling and CVD.

Another major limitation is the possibility to use this biomarker in the daily practice. The computational step and the need to measure three biomarkers make this approach not easy to implement and potentially expensive. As with other novel biomarkers it is however possible that measurement of the B/T ratio might be advocated to guide treatment in selected groups of individuals and patients, as for example patients with chronic autoimmune disorders or as suggested in Study II in those with low-normal LDL cholesterol levels.

On the other hand, the importance of creating an "integrated" biomarker is suggested by the data reported in Study I. We observed, in line with previous studies, that IL6 and sIL6R displayed a linear association (26, 148-151, 216). Hence, both IL6 and sIL6R are markers of increased risk, albeit only the two in combination or sIL6R alone mirrors IL6 trans-signalling exclusively. IL6 signalling is causally related to CAD via the genetically determined shedding of sIL6R (142, 217). In context of what we know of IL6 biology and together with the existent genetic and clinical data, the results from Study I suggest that the binary IL6:sIL6R complex is the active component rather than IL6 itself. Hence both entities are

needed to assess the activity in the pathway (83, 142, 148, 150-152). The picture is however not complete until sgp130 is added. In our study, sgp130 displayed a non-linear association and in previous observational studies, sgp130 has been associated with both protective and detrimental properties (114, 151, 153, 154). While administering supra-physiological doses of recombinant sgp130Fc resulted in dampened atherosclerosis and fewer ruptures of abdominal aortic aneurysms in two murine models of atherosclerosis (138, 140). Considering the above, physiological sgp130 levels possibly mirrors the activity in the pathway rather than the level of IL6 trans-signalling inhibition. As the ability of sgp130 to inhibit IL6 trans-signalling is completely dependent on the presence of the binary IL6:sIL6R complex, the protective effect of physiological sgp130 cannot be evaluated on its own. Moreover, we cannot exclude that sgp130 levels mirror other inflammatory processes independent from the IL6 trans-signalling. For instance, IL11 trans-signalling is inhibited by sgp130Fc and in theory might also be inhibited by circulating sgp130 (218).

Overall, we observed a strong and consistent association between the B/T ratio and the risk of CVE in line with previous studies on the association of the sIL6R with CVE risk (148-152). We then tested it the B/T ratio could improve the prediction of CVE. The measures of discrimination and re-classification were slightly increased which is in line with prior studies since these are models constructed for evaluating diagnostic and not predictive tools (219). In the models, we used the FRS as the reference and not the European SCORE which has cardiovascular mortality as outcome (8). In addition, IL6 was used instead of CRP as we wanted to analyse the potential improvement in prediction with the B/T ratio in relation to an existent risk markers albeit did not want to use CRP as it is not part of the trans-signalling pathway (220).

5.2 Study II

In Study II, we found that the risk of CVE associated with the B/T ratio >median was higher in subjects with LDL ≤4.0 mmol/L compared to those with higher LDL levels and in those with low-intermediate risk according to the FRS. This is the group of people where novel biomarkers are mostly advocated and those who potentially would benefit the most from the B/T ratio as a predictor of future CVE. Above all, in the intermediate cardiovascular risk group the assessment of potential re-classification upwards or downwards is of great importance to address the possible need for intervention (221).

In addition, individuals with a B/T ratio >median experienced a higher CVE/CAD/ischemic stroke risk and earlier events than those with lower B/T ratio and this was primarily evident among those with low LDL levels.

In spite of new guidelines, such as the European Guidelines on cardiovascular disease prevention in clinical practice from 2016, emphasising the need to risk stratify individually, patients are still treated in a secondary preventive manner resulting in insufficient preventive treatment for individuals without a first-ever CVE but a high cardiovascular risk (222).

To assess the risk associated with the B/T ratio, in individuals at low-intermediate and high cardiovascular risk, we chose to stratify by LDL with a cut-off of 4.0 mmol/L. The 4.0 mmol/L cut-off gave us the best sensitivity and specificity for LDL as a predictive marker of CVE in our cohort. Harmonising with other studies, the cut-off was in line with levels re-classifying between risk groups in the FRS and in a Swedish cohort study (208, 223). The association was also tested stratified by the FRS <10%, 10-20% and >20% 10-year CVE risk and found to be a predictive marker in those at low-intermediate risk. These findings are in line with studies of other cardiovascular biomarkers displaying a greater discriminatory potential in subjects at low risk (221, 224).

The more recent guidelines introduce a cut-off of 3.0 mmol/L for LDL cholesterol as target for eventual treatment with cholesterol lowering agents (225). Our data suggest that even with LDL levels lower than 4.0, the B/T ratio still predicts CVE. However, due to the small number of cases in the group with LDL <3.0 we could only demonstrate a trend similar to that of the analysis with the higher cut-off.

In the biology of atherosclerosis, the growth of the atherosclerotic plaque is dependent on the interaction between oxidised-LDL, foam cells producing inflammatory mediators and anti-inflammatory cytokines. In our study we tested the presence of a biological interaction between LDL and the B/T ratio. We have used a biological interaction model on an additive scale: this model can be used if there is a strong biological hypothesis underlying a possible interaction between the two exposures. We observed that the effect of IL6 trans-signalling on the CVE risk was independent from that of LDL cholesterol when analysing the interaction between the two. The measure of interaction in fact did not show a significant increase in CVE risk in those with both high LDL levels and B/T ratio. An important consideration is that the absence of a statistically significant interaction may be due to lack of power. Therefore, these results can only be considered as hypothesis generating. One implication of this result, if confirmed in other populations, is that targeting the IL6 trans-signalling may have an effect which is independent from the one obtained targeting LDL with lipid lowering agents. To this extent, targeting the IL6 trans-signalling may achieve a different anti-inflammatory effect from that observed during statin treatment resulting in lower CRP levels.

5.3 Study III

In the first ever study analysing the local expression of IL6 signalling in human atherosclerotic plaques, we found that all components of IL6 signalling are present in carotid plaques and that their expression seem to differentiate depending on the severity of the disease and statin treatment. Of importance, plasma sIL6R correlated with plaque expression of *sIL6R*.

Plaques from patients who had suffered a cerebrovascular event ≤6 months prior to surgery displayed significantly higher expression of *IL6R* compared to asymptomatic patients. Furthermore, expression analyses indicated higher *sIL6R* and lower *GP130* and *sGP130* expression in symptomatic patients. These findings confirm the local presence of IL6 signalling in carotid plaques and the pattern of differential expression suggests a possible association between local gene expression and plaque instability mirrored by the recent cerebrovascular event. In line with our findings, previous analyses of plaque gene expression in the BiKE study demonstrated upregulation of inflammation associated genes in symptomatic subjects (205). Our findings are however suggestive as our cohort is small lack of statistical significance cannot be ruled out as absence of association. In a recent *in vitro* study on hepatocytes with a computational modelling approach, it was proposed that IL6 trans-signalling is the dominant IL6 pathway when gp130 protein expression exceeds IL6R expression (226). Given our transcriptomic approach and experimental design we cannot draw any conclusions on which of the two IL6 pathways is more active. Nevertheless, the results of the gene expression analysis indicate a higher absolute expression level of *GP130* compared to *IL6R* regardless of the symptomatic/asymptomatic status of the patient. In addition, upregulated *GP130* expression could be indicative of IL6 trans-signalling in line with the positive autocrine feedback loop described on vascular SMC (135).

To assess if peripheral circulating levels of the IL6 signalling components mirror local activity in the IL6 pathways, we found that plaque expression of *IL6* and plasma IL6 and plaque *sIL6R* and plasma sIL6R, respectively were positively correlated. Moreover, plasma sIL6R was the only marker that decreased with time from the cerebrovascular event to CEA indicating that the sIL6R could be used as a predictive biomarker of atherosclerosis and possibly also to monitor inflammatory activity locally in plaques.

In additional analyses, the plaque expression and plasma levels were analysed with regards to whether or not the patient was on statin treatment. Here we found that *sIL6R* and *sGP130* were expressed to a greater extent in plaques and plasma sIL6R was higher in statin-treated patients compared to non-treated. These findings of a potential upregulation of the IL6 trans-signalling components when subjected to statins are in line with our previous findings of the B/T ratio as a stronger predictor in individuals with low-normal LDL cholesterol levels.

With these findings we conclude that IL6 signalling is present locally in carotid artery plaques. These results also suggest that circulating levels of the binary IL6:sIL6R complex could be used as a marker of prevalent atherosclerosis.

5.4 Study IV

Study IV concluded that the B/T ratio is associated with ischemic stroke in individuals without prevalent or incident AF while this association could not be demonstrated in subjects with AF. In secondary analyses, we found that there was no association between the B/T ratio and risk of incident AF in individuals free of prevalent or incident CVD.

Ischemic stroke is a well-defined clinical condition resting on heterogeneous and complex pathophysiological ground making epidemiological studies on ischemic stroke challenging. In Study II, the B/T ratio >median was associated with the risk of ischemic stroke and earlier cerebrovascular events, albeit we had not stratified by ischemic stroke type. To further analyse this association, we explored the presence of IL6 signalling in carotid plaques in Study III and found a pattern of suggestive differential gene expression depending on degree of plaque stability.

In Study IV, we were therefore curious to see if IL6 trans-signalling was primarily associated with atherothrombotic stroke or if there was also an association with AF and cardioembolic stroke. The inflammatory mechanisms in atherosclerosis of the carotid arteries are to a large extent similar to those in the coronary vessels while the environment in the intracerebral vasculature, not covered in this thesis, is much different owing to the cerebral blood pressure autoregulation, blood brain barrier and immune cells exclusive to the CNS (10).

Given that sIL6R and sgp130 are produced through post-transcriptional mechanisms, genetic studies demonstrating an association between *IL6R*, AF and ischemic stroke cannot differentiate between IL6 classical and IL6 trans-signalling but merely establish the association with IL6 signalling in general (194, 195). To explore the association between IL6 signalling and AF we analysed the association with each component of IL6 trans-signalling and found an association only with high levels of circulating IL6. Considering the increasing evidence of cardiac remodelling with atrial cardiopathy as a plausible mechanism for cardiac thromboembolism in AF, our results could potentially be explained by increased IL6 classical signalling known to be involved in vascular remodelling and in cardiac hypertrophy induced by angiotensin II (81, 180, 183, 227).

Our results clearly suggest that IL6 trans-signalling is associated with atherothrombotic ischemic stroke while the association to AF and AF in relation to ischemic stroke could not be demonstrated.

In summary

Although the studies in this thesis prevent us from drawing any mechanistic conclusions, our results are in line with what is known about the IL6 signalling biology and the results from existing clinical studies. With these findings we conclude that IL6 trans-signalling, estimated by the B/T ratio, is a potential predictive biomarker of future CVE, primarily in individuals with low-intermediate cardiovascular risk. Moreover, the presence of the signalling moieties in carotid artery plaques together with previous experimental mouse studies suggest that IL6 trans-signalling could be a novel target for immunomodulatory treatment of manifest atherosclerotic CVD and the B/T ratio could possibly be used to monitor treatment.

5.5 Methodological considerations

5.5.1 Study design

All studies in this thesis are observational and thus cannot contribute with mechanistic evidence.

Study I, II and IV were performed in the 60YO, a large prospective population-based cohort with a high positive response rate and complete follow-up of the outcome in national registers which diminishes the risk of selection bias. Conducting cohort studies with follow-up by means of national registers is effective, inexpensive and secures follow-up. In addition, prospective cohorts have the advantage that temporality can be assessed i.e. the exposure precedes the outcome.

A limitation of the study design of the 60YO is the fact that blood sampling and gathering of information on e.g. medication only was performed at baseline thus preventing the assessment of variations in biomarker serum concentrations and changes in medication during follow-up. The single baseline measurement of the biomarkers could introduce a misclassification of the exposure. We have however only included participants without prevalent CVD at baseline. MI and ischemic stroke are preceded by several years of silent CVD albeit we have no knowledge of mechanisms inflicting a greater variation in cytokine and receptor levels in future cases of CVE than in referents. However, subjects with subclinical CVD could possibly have a greater variability in serum levels during follow-up due to low-grade chronic inflammation in the vessels. If so, the potential misclassification would be differential and possibly affect the risk estimate.

In addition, at the time Study I and II were performed we did not have the ethical permission to retrieve diagnoses of other prevalent or incident non-cardiovascular outcomes from national registers in the 60YO. This has prevented us from analysing the risk associated with the B/T ratio in other disease where inflammation plays an important role as well as the competing risk with CVD.

In Study III, we have analysed a sample from the BiKE study where we could assess all the components of the IL6 trans-signalling in both plasma and tissues. The sample size was however small possibly explaining the lack of statistical power to analyse the differentially in gene expression and correlations between gene expression and plasma levels. Moreover, the analyses were cross-sectional preventing temporality from being addressed. The major strength of Study III was that it was performed in a real-world setting of consecutive patients amenable for CEA in Stockholm 2002-2007 thus increasing potential generalisability and minimising selection bias.

5.5.2 Misclassification of the exposure

5.5.2.1 Biomarker measurements

Circulating levels of IL6, sIL6R and sgp130 were measured in serum (60YO) or plasma (BiKE) using the same assays in all four studies. Similar for both studies, serum/plasma samples had been kept for several years in a large biobank frozen to -80 degrees Celsius until the measurements, underlying the biomarker data in this thesis, were performed.

This phenomenon is not unusual in epidemiological biomarker research albeit has not been specifically investigated. If concentration levels were supposedly lower or higher due to long storage this would however lead to non-differential misclassification as neither the exposure (IL6 trans-signalling) or the outcome (CVE) would affect the degradation differentially. When compared to the existing literature, our results are in line with previous cytokine-based studies both in terms of direction and size of the association (148, 150-152). Furthermore, we do not aim to establish an absolute cut-off for any of the biomarkers studied.

The commercial assays used to analyse IL6, sIL6R and sgp130 do not discriminate between free and bound molecules. We have incorporated the individual components of IL6 trans-signalling into the B/T ratio based on a previously described formula hence the proportion free vs. bound IL6 and sIL6R is not of interest. Moreover, in the analysis of serum and plasma sgp130 we could not discriminate between different isoforms of sgp130 albeit when we calculated the molar concentration of sgp130 we assumed a molecular weight of 100 kD (75), under the assumption that the most common form of circulating sgp130 is the full length, whose molecular weight is about 100 kD. In Study III, we have not calculated the B/T ratio since prospective data were not available to estimate the future risk of CVE in this population and we could not correlate the intra plaque B/T ratio with the circulating one.

5.5.2.2 Gene expression analyses

In Study III, gene expression of *IL6*, *IL6R*, *sIL6R*, *GP130* and *sGP130* was analysed in carotid plaques extracted and cut in half during CEA with the one half (most proximal) being prepared by the BiKE group researchers for RNA analysis. When analysing expression in specific parts of the plaque there is a risk of missing receptors if there is an uneven distribution of receptors within the plaque. To overcome this risk, the plaque can be homogenised which was not done in the present study. Another limitation is that inflammatory markers in the plaques could be affected by the surgery through which the plaque was obtained. This limitation is however impossible to overcome as all access to atherosclerotic plaques in living humans will entail a surgical procedure. Moreover, changes in the proportion of inflammatory cytokines within the plaque could take place after extraction from the vessel. To overcome this risk, plaques were immediately frozen to -80 degrees Celsius in connection to surgery.

Given the post-transcriptional modifications of the *IL6R* and *GP130* genes, resulting in the sIL6R and sgp130 proteins, it would have been of great value to have done proteomics in carotid plaques instead of or in addition to transcriptomics. This limitation could result in differential misclassification as shedding is to a large extent driven by inflammation and patients with a recent plaque rupture would be expected to be subject to local inflammation in the carotid artery with increased frequency of shedding. Given that we saw significantly higher *IL6R* gene expression in plaques from symptomatic patients and that plasma levels of sIL6R decreased with time from cerebrovascular symptom to CEA this could imply that in the acute inflammatory context of a plaque rupture, shedding might increase circulating sIL6R and enabling IL6 trans-signalling. We did however want to ensure that the receptors quantified in the plaques were synthesised in the plaque and not up taken from the circulation and for this reason we decided to explore the gene expression in this first study of IL6 signalling in carotid plaques.

With regards to *sGP130* expression, only *sGP130-RAPS* was quantified as it is the sole sgp130 isoform verified by Western blot (94). Given that full length sgp130 is the most frequent circulating sgp130 isoform and in addition the most potent one this is a major limitation. Given our experimental design, we could not co-amplify more than two isoforms for each gene. For this reason, we chose the isoform whose transcription had been verified. In addition, the IL6 receptors were co-amplified in pairs (*IL6R*; *sIL6R* and *GP130*; *sGP130*) hence any potential differences in the expression levels between the membrane-bound (IL6R and GP130) and soluble receptor (sIL6R and sgp130) cannot be analysed.

Finally, we chose the average between the expression levels of two housekeeping genes to normalise our expression analysis. The reason underlying this choice was that we wanted at least two housekeeping genes given the complexity of the tissue analysed.

5.5.2.3 Residual and unmeasured confounding

Increased levels of IL6 and its receptors could mirror other inflammatory conditions that we have not analysed and hence could not adjust for. The population we analysed, was relatively homogenous being from the same generation living in the capital of Sweden, however other important factors such as socio-economic factors were not taken into account in this thesis.

5.5.3 Misclassification of the outcome

All information of the outcome is derived from the Swedish national health registers: The National Inpatient Register and the Cause of Death Register, using ICD-10 diagnosis codes, introduced in 1997 in Sweden. The positive predictive value of diagnoses in these registers is high and they are deemed reliable (196). As the National Inpatient Register does not include diagnoses from primary care we will have missed to categorise some individuals as cases (228). This is above all a problem when it comes to AF as MI and ischemic stroke in the absolute majority of cases will be cared for in hospitals. In Study IV, the secondary outcome was AF and we have accounted for the lack of primary care diagnoses by retrieving both main and secondary diagnoses of AF from the inpatient register. Another problem with retrieving diagnoses from registers is that some diagnoses such as AF often entail a delay in diagnostics due to subtle symptoms early in the course of the disease. There is hence a risk of misclassification of the outcome here and with that a possibility of underestimating the risk of AF associated with the B/T ratio, since a certain proportion of AF case would be falling in the referent group moving the HR towards 1. The same risk of diluting the association would however apply for IL6 which exhibited an association with AF. Possibly then either our findings correctly exclude an association of the B/T ratio with AF or the association is weaker than that observed with IL6.

5.6 Conclusions

I IL6 trans-signalling estimated by the binary/ternary complex ratio, the B/T ratio, is associated with the risk of future CVE in individuals without prevalent CVD.

II The B/T ratio is associated with the risk of future CVE and early CVE primarily in individuals with a low-intermediate cardiovascular risk defined by LDL cholesterol or the Framingham Risk Score.

III The genes of all components of the IL6 signalling pathways (*IL6*, *IL6R*, *sIL6R*, *GP130*, *sGP130*) are expressed in carotid artery plaques in patients with significant carotid artery stenosis.

IV The B/T ratio is associated with the risk of future atherothrombotic but not cardioembolic ischemic stroke. The B/T ratio is not associated with incident AF in individuals without prevalent or incident CVE.

5.7 Future perspectives

IL6 trans-signalling is associated with atherosclerosis progression and the clinical manifestations thereof. The CANTOS and COLCOT studies, demonstrated the need for inhibition of the IL1β-IL6-CRP pathway albeit with increased serious adverse events most likely due to the concomitant inhibition of IL6 classical signalling and resulting deficient immune response (12, 215). Some researchers advocate moving upwards in the pathway, to antagonise the inflammasome (229). As seen in **Figure 21**, inhibiting the IL1β-IL6-CRP pathway upstream from IL6 trans-signalling will however inevitably affect IL6 classical signalling.

Apart from its relevance as biomarker of CVE risk, targeting the IL6 trans-signalling might thus represent a novel and safer therapeutic moiety. Compared to targeting IL6 or upstream of IL6, selective inhibition of IL6 trans-signalling is likely not to induce the observed adverse effects such as neutropenia and fatal infections (12, 119, 215).

Dampening IL6 trans-signalling with recombinant sgp130Fc has been shown to be effective in experimental mouse models of atherosclerosis and is currently tested in a randomised controlled trial in IBD (124). Given that the result is positive, the next step could be a randomised controlled trial of sgp130Fc in patients with stable CAD and/or ischemic stroke. To select suitable participants most likely to benefit from treatment, the B/T ratio could be used both as a marker of increased IL6 trans-signalling and further, during treatment, as a biomarker to monitor treatment effects.

6 SVENSK SAMMANFATTNING

Samspelet mellan cirkulerande inflammatoriska molekyler och kärlväggens celler orsakar en kronisk inflammation som leder till åderförkalkning (ateroskleros) med hjärninfarkt (stroke som orsakats av en propp) eller kranskärlssjukdom ledande till kärlkramp eller hjärtinfarkt som följd. En modern klinisk läkemedelsprövning visade nyligen att behandling med en antikropp som hämnar inflammationen vid ateroskleros minskar risken för nya hjärtkärlhändelser efter hjärtinfarkt. Man behöver dock utveckla nya metoder för att identifiera vilka patienter kan gynnas av en sådan anti-inflammatorisk behandling.

Syftet med denna avhandling är att undersöka om den inflammatoriska interleukin 6 (IL6) trans-signaleringsvägen är associerad med ökad risk för hjärtkärlhändelser (svår kärlkramp, hjärt- och hjärninfarkt) och i så fall om en markör för IL6 trans-signalering kan användas för att predicera en ökad risk för hjärtkärlhändelser i framtiden hos individer utan känd hjärtkärlsjukdom samt att belysa IL6 trans-signaleringens roll vid etablerad ateroskleros.

I studie I, II och IV har vi använt oss av 60-års-kohorten, en studie där var tredje 60-åring boendes i Stockholm 1997–1999 slumpvis utvaldes och tillfrågades om att medverka. Vid studiestart fick de 4232 deltagarna fylla i frågeformulär om livs-stilsfaktorer, sjukdomar och läkemedelsbehandling. Blodprover togs vid studie-start och med dessa har det aktiva binära IL6 komplexet och det inaktiva tertiära komplexet analyserats och sammanställts i en kvot, den så kallade B/T kvoten. I vår studie inkluderades endast hjärtkärlfriska studiedeltagare och dessa har sedan följts i svenska hälsoregister för att se vilka som insjuknat i hjärtkärlhändelser under uppföljningstiden (studie I-II t.o.m. 2014, studie IV t.o.m. 2017).

I **studie I** undersökte om vi om B/T kvoten var associerad med risken för framtida hjärtkärlhändelser och kunde visa att deltagare som hade en hög B/T kvot, dvs en hög proportion av det aktiva binära komplexet i förhållande till det inaktiva tertiära komplexet, hade en ökad risk för framtida hjärtkärlhändelser.

I **studie II** ville vi undersöka om B/T kvoten var mer lämplig som markör i vissa grupper. Med tanke på att patienter som har en hög risk för framtida hjärkärl-händelse av andra orsaker så som höga blodfetter eller många hjärtkärlriskfaktorer får en grundlig genomgång och behandling ville vi undersöka om B/T kvoten kunde vara en lämplig markör för en ökad hjärtkärlrisk hos individer som inte hade en ökad risk värderat med de instrument vi har idag. Vi testade därför om B/T kvoten var en lämplig markör hos de som hade låga-normala LDL-kolesterolnivåer eller de som med Framingham riskscore (internationellt validerat formulär för att mäta hjärtkärlrisk) hade förväntat låg risk för framtida hjärtkärlhändelser. Vi kunde visa att B/T kvoten var mer effektiv att predicera risk för hjärtkärlhändelser hos dem

som hade lågt LDL-kolesterol eller en förväntat låg hjärtkärlrisk med Framingham riskscore. Dessutom kunde vi i denna grupp av individer predicera tidiga hjärtkärlhändelser, framför allt hjärninfarkt. Vid test av interaktion kunde vi se att B/T kvoten och LDL-kolesterol inte verkade påverka risken tillsammans utan var för sig.

I **studie III** ville vi undersöka om signalmolekylen IL6 och de receptorer som gör att IL6 kan påverka inflammationen i kärlen kunde hittas vid analys av gener i åderförkalkningsplack från förträngningar i halskärlen hos 78 patienter som opereras för denna förträngning. Vi analyserade gener i åderförkalkningsplacken tillsammans med nivåer av IL6 och dess receptorer i blodplasma i en kärlkirurgisk studie, the Biobank of Karolinska Endarterctomies (BiKE). Av de 78 patienterna hade 53 haft en hjärninfarkt eller TIA, dvs strokesymptom som gått över inom ett dygn, inom de senaste 6 månaderna. Åderförkalkningsplacken hos dessa patienter betraktades som instabila om symptomen kunde stämma med att det var en propp från kärlet som orsakat hjärninfarkten/TIAn. Vi kunde se att IL6 och all dess receptorer fanns i åderförkalkningsplack i halskärlen på patienterna i BiKE studien. Vi såg också tecken till ökad mängd av de gener som ingår i det aktiva binära komplexet i plack hos patienter som haft en nylig hjärninfarkt/TIA.

Då vi i de föregående studierna sett att IL6 trans-signalering verkar vara associerat med hjärninfarkt och speciellt av den typen som beror på en propp från ett åderförkalkningsplack i ett förträngt halskärl så ville vi i **studie IV** undersöka om det också fanns en koppling till den typ av hjärninfarkt som orsakas av den vanliga hjärtrytmrubbningen, förmaksflimmer, känd för att kunna orsaka hjärninfarkt. Vi testade om B/T kvoten var associerad med risken för framtida hjärninfarkt hos individer med och utan känt förmaksflimmer i 60-års-kohorten och fann att B/T kvoten endast var associerad med hjärninfarkt hos individer utan förmaksflimmer. Vidare testade vi om B/T kvoten var associerad med förmaksflimret i sig men fann att den inte var det.

Slutsatsen av denna avhandling är att IL6 trans-signalering mätt med B/T kvoten är associerat med en ökad risk för framtida hjärtkärlhändelser och att B/T kvoten därför kan användas som markör för ökad risk framför allt hos individer som bedöms ha en låg risk med nuvarande prognosinstrument. Vidare är B/T kvoten en god prediktor för framtida hjärninfarkt men verkar inte kunna predicera risken för hjärninfarkt orsakad av förmaksflimmer eller risken för förmaksflimmer ensamt.

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